MAR 3 1921

Vol. II. No. 1] [February, 1921.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

Editors:

C. H. BROWNING
C. L. EVANS
P. FILDES
W. E. GYE
E. L. KENNAWAY
E. H. KETTLE
J. McINTOSH
J. A. MURRAY
W. J. TULLOCH
C. M. WILSON

With the Support of:

F. W. ANDREWES (London)
W. M. BAYLISS (London)
W. BULLOCH (London)
S. L. CUMMINS (London)
H. H. DALE (London)
A. M. DRENNAN (Otago, N.Z.)
J. W. H. EYRE (London)
LEONARD FINDLAY (Glasgow)
DUNCAN GRAHAM (Toronto)
R. T. HEWLETT (London)
F. G. HOPKINS (Cambridge)
W. B. LEISHMAN (London)
J. J. MACKENZIE (Toronto)
T. J. MACKIE (Cape Town)
H. GERTEL (Montreal)
LEONARD ROGERS (Calcutta)
C. S. SHERRINGTON (Oxford)
A. E. WRIGHT (London)

LONDON: H. K. LEWIS & CO. LTD., 136, GOWER STREET, W.C.1.

EDITORIAL AND PUBLISHING OFFICES: 23, GOWER PLACE, W.C.1.

CANADA: THE MACMILLAN COMPANY OF CANADA, LTD., BOND STREET, TORONTO.

Annual Subscription, post free, Two Pounds net.

Single Numbers, Seven and Sixpence net.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

EDITORIAL NOTICE TO CONTRIBUTORS.

Scope of Papers.—This Journal has been founded for the publication of original communications describing the technique and results of experimental researches into the causation, diagnosis and cure of disease in man. Papers upon bacteriological, biochemical, physiological, serological and other subjects are therefore acceptable when bearing directly upon these objects.

A section will be devoted to short communications upon new technical methods applicable to those subjects which come within the scope of the Journal.

It is also the intention of the Editors to publish occasional "critical reviews" upon pathological subjects which may at the time be in need of critical survey. Such reviews may be of value to workers who are not in touch immediately with considered opinions upon the questions discussed.

Forwarding Papers.—Papers should be addressed to—

THE EDITORS,
British Journal of Experimental Pathology,
28, GOWER PLACE,
LONDON, W.C. 1,

or to any of the Editors personally.

Date of Publication.—The Journal is published bi-monthly, in February, April, June, August, October, and December.

Priority of Publication.—Papers accepted for publication will be printed as far as possible in the order of their receipt by the Editors, but those sent from overseas will receive priority in publication according to the date of posting instead of the date of receipt.

Conditions of Publication.—Contributors should prepare their manuscripts with the greatest attention to detail. The fact of submitting a paper for publication will be taken by the Editors as an admission by the Author of his liability to pay for all unreasonable alterations made by him in proofs, and for mistakes due to lack of clearness in the preparation of the manuscript.

Illustrations and charts will be accepted for publication, but if, in the opinion of the Editors, they are not required to increase the clarity of the argument, they must be paid for by the Authors.

References.—Authors are requested to prepare their references according to a systematic plan, namely, as given in the *Index Catalogue of the Surgeon-General's Library* at Washington. They should be numbered consecutively in the text, and collected at the end of the paper in the same order under "References." The initials, title, year, volume and page should all be included.

Proofs.—To avoid delay, proofs will not be submitted to Authors overseas unless they express a desire to see them, or unless the Editors think it necessary. Overseas Authors are therefore particularly reminded of the importance of preparing their manuscripts in such a way that error need not arise.

Reprints.—25 reprints will be supplied free to authors. Additional copies must be ordered from the Publishers *before the issue is printed*, and will be charged for according to the following scale:

		£ s. d.	£ s. d.	£ s. d.	£ s. d.
Number of Additional Reprints	25	1–4 pp., 0 11 6	5–8 pp., 1 2 6	9–12 pp., 1 13 6	13–16 pp., 2 4 6
	50	,, 0 13 9	,, 1 6 8	,, 1 19 0	,, 2 12 0
	75	,, 0 16 0	,, 1 10 6	,, 2 4 6	,, 2 19 6
	100	,, 0 18 3	,, 1 14 6	,, 2 10 0	,, 3 7 0

Authors working in countries where the rates of exchange are unfavourable to them will be charged for reprints according to pre-war rates (25 francs = 20 shillings).

THE BRITISH JOURNAL
OF
EXPERIMENTAL PATHOLOGY

THE MEASUREMENT OF CAPILLARY BLOOD-PRESSURE IN MAN.

LEONARD HILL, F.R.S.,

National Institute for Medical Research, Hampstead, London;

AND

JAMES M. McQUEEN.

Received for publication December 17th, 1920.

An accurate measurement of capillary blood-pressure in man is much to be desired, but difficult of attainment. It is true that measurements are to be found in every text-book, and equally true that these measurements are tacitly, at least, applied as accurate to other physiological problems, such as the origin and production of lymph. Physiologists have not hesitated to accept the current measurements, correlated them with measurements of the osmotic pressure of colloid, such as blood-plasma, and found a balance of pressure in the capillaries in favour of filtration or other purely physical theory of the origin of lymph. No less, in the theories of the production of urine, capillary blood-pressure measurements are made of fundamental importance.

Measurements of capillary blood-pressure in man were first attempted by V. Kries, using a glass plate on the terminal phalanx of the finger below the finger-nail. By placing weights on a pan attached to the plate, and calculating the surface area of the plate and the weight which just sufficed to blanch the area below the glass, measurements of capillary blood-pressure varying, of course, at different levels from the heart were obtained ranging from 30–40 mm. Hg. and over.

It was assumed in these measurements that the area comprised a more or less uniform capillary field, and that the resistance of the skin was not a factor of much importance.

It is unnecessary to enter into the details of other methods of achieving capillary blood-pressure measurements in man, as the data, no matter what

method is employed, do not materially differ from those given by V. Kries. Thus, recently, Danzer and Hooker (1) have microscopically observed the flow in the skin adjacent to the nail, using a suitable chamber into which air was pumped so as to stop the flow, and a manometer to measure the pressure. They found in eighteen individuals the stopping-pressure averaged 18–26·5 mm. Hg.

It was, however, shown by one of us (L. H.) (2) that if a hair be taken which will just blanch the skin when pressed on it till it bends, a weaker hair will suffice after shaving off the outer horny layers of the epidermis.

The difficulty in securing a uniform capillary field in man on which observations might be made, it is safe to say, will hardly be overcome. The arrangement of arterioles, capillaries and venules as a meshwork prevents the absolute isolation of any component of the mesh. In the case of the frog's web an area of 2 mm. in diameter includes all these vessels. On compression of such an area blanching occurs only when the systolic pressure in the arterioles supplying the area is overcome, as may be seen by observation of the frog's web compressed by Roy and Graham Brown's method (3). The wider the area chosen the larger may be the arterioles compressed and the higher may the readings be. Accordingly a method of compression should be chosen so that a minimal area is compressed. Further, if the compression could be made momentary, it might express blood and blanch the area before the pressure had time to bank up to the systolic pressure in the arteries which supply the arterioles of the compressed part.

Observations made by one of us (L. H.) (4) on the frog's web and mouse's ear and mesentery show that 2–5 cm. of water is the pressure which, when quickly applied, momentarily checks the flow in the arterioles and their main capillary branches, and that the checking and not the stopping pressure, which may be 35 cm. of water, gives the true index. Unfortunately it is not possible to secure such a momentary index of compression in man, so the readings are bound to be too high. The venous pressure in the main veins of the arm as measured by observing the collapse of the superficial veins on raising the arm above the heart-level is some 3–10 cm. of water. Now the venule-pressure closely approximates to that in the capillaries in the case of the frog and mouse.

Some twenty-five years ago the capillary pressure in the brain of the dog was measured by one of us (L. H.) (5) by finding the pressure required just to flatten that part of the brain which bulged out into a trephine hole. A tube closed by a membrane was screwed into the trephine hole so that the membrane came into contact with the brain. The tube was filled with water and connected with a glass tube which contained an air-bubble index, and this in its turn with a manometer and pressure bottle. The air index was forced out from the zero position by the brain, was brought back to this position by raising the pressure bottle and the pressure indicated by the manometer then read. This method was applied (in the case of a man) to a trephine hole covered with skin, a special contrivance being used to secure an air-tight connection of the apparatus with the shaved skin in which the principle of the cupping glass was used. The capillary pressure in the brain, with the man in the horizontal position, by such a method was found to be a very few millimetres of mercury. It was in the dog found to be the same as the pressure in

the venous sinuses and as that of the cerebro-spinal fluid. It varied with the rise of venous pressure due to expiratory effort, with rise of arterial pressure and with change of posture. In the erect posture it might be at times zero or even negative. In the infant exhausted by illness the anterior fontanelle is depressed. On the other hand, in a fit of crying in the case of a lusty infant it bulges out and feels tense. In the spasm produced by strychnine, or essential oil of absinthe, the pressure in the capillary vessels, which is the same as the pressure of the brain against the skull-wall, might rise to 40–50 mm. Hg., owing to the great increase of arterial pressure, coupled with an obstruction of the venous return. As has already been stated, the capillary pressure of the brain was found to be the same as that of the cerebro-spinal fluid. In a case of hydrocephalus the pressure varied with the compression of the head by the observer's hands and became zero when the fluid was aspirated off.

So, too, in the case of the eye, wherein the capillary pressure is regulated by the secretion pressure of the aqueous fluid, the pressure of the fluid outside and the blood inside the capillaries always balance one another. Thus it was shown by Hill and Flack (6) that on raising the pressure in the aqueous chamber the circulation in the eye continued, as shown by the outflow from a vortex vein, until the arterial pressure (measured in the carotid) was just overtopped, *i. e.* the pressure in the capillaries and veins rose *pari passu* with that of the fluid in the aqueous chamber. It was also shown that when the aqueous fluid was allowed to escape the iris bulged forward owing to the pressure in the capillaries no longer being balanced; in such a case a compression of the abdomen, through raising the venous pressure, easily burst these vessels in the iris and caused hæmorrhage. Struggling during an operation on the anterior chamber of the eye may have the like effect on man.

In the case of the salivary gland of the dog, Hill and Flack (7) also showed that when the secretory pressure rises above the arterial pressure, the outflow from the salivary vein continues. They pointed out that the cells of each alveolus are enclosed by a *membrana propria* which limits their expansion, so that the venules can be compressed, but not shut up as the secretory pressure rises. Owing to arterial dilatation and compression of the veins and venules by the swollen alveoli the pressure rises in the veins and capillaries, and the blood-flow becomes a rapid one through the whole vascular system of the gland, which is approximate to a rigid system. The tenseness of the whole gland can then be felt by the finger. Similarly in inflamed parts the tension and throbbing are due not only to arterial dilatation, but also to the rise of imbibition pressure within, and swelling of the tissue cells, with consequent compression of veins, this in its turn leading to the vascular pressure in the inflamed part approximating throughout capillaries and venules to arterial pressure. The sequence of the phenomena is exactly similar to that which occurs when the arm is compressed by the cuff of the sphygmomanometer, a sequence which may be observed under the microscope in the frog's web, lung, etc., when compressed by the membrane of an apparatus similar to that devised by Roy and Graham Brown for measurement of capillary pressure.

In the case of man, Hill and Flack (7) found that it took but a very few centimetres of water-pressure to drive fluid under the skin through a hypo-

dermic needle, and the conclusion was reached that capillaries must be exsanguined when water is so driven in, and that the pressure required to effect this is very low under normal resting conditions. When the arm is compressed by the cuff of the sphygmomanometer with a pressure just below that in the artery, the pressure in the superficial veins rises up to this pressure (?), but it does not then take anything like this pressure to drive water under the skin of the arm. The veins are filled through the wider anastomoses while many capillaries are still largely not filled to congestion. In a case of œdema of the leg a hollow needle indicated a pressure of a few centimetres of water when the leg was placed at the same level as the heart, Compression of the leg by the hands at once raised the pressure, and it is obvious that capillary pressure must rise *pari passu* with that of the œdema fluid for the circulation to continue. The conclusion was reached that the capillary pressure is largely governed by the imbibition pressure of the tissue-cells, vessels and cells being both confined by the membranes and skin of the body.

In the eye the secretory pressure of the aqueous fluid regulated by the cells of the ciliary processes fixes the pressure at some 25 mm. Hg., while in the brain the secretory pressure of the cells of the choroidal fringes fixes the pressure at some 5 mm. Hg., the subject being in the horizontal position and resting. With rise and fall of venous, arterial or secretory pressure, tissue fluid and capillary pressures vary together.

In the skin of the healthy person the pressures are kept just positive, giving the fulness to the countenance. The face looks drawn when the imbibition tone of the tissues falls and the circulation fails to keep the capillaries distended, *e.g.* in acute diarrhœa.

To try and arrive at a juster measure of the pressure in the capillaries in man, one of us (J. McQ.) has designed the following method :

If water from a jet fed from a reservoir kept at constant pressure-head be played on the skin below the nail or on the skin covering the distal end of the metacarpal bone of the thumb, the jet having a 2 to 2·5 mm. bore diameter, it will be noted that if the pressure be sufficiently high, the area impinged on can be rendered white ; when the jet is removed a red blush of returning blood is seen. The horizontal jet of water striking the thumb being dispersed by impact tends to reflect light, and if we look into the jet the skin below appears whitened ; such whitening must not be taken as the index. Moving the jet quickly to one side and watching the area just impinged on by the jet of water we note the return of the blood into that blanched area. The pressure which just suffices to produce blanching is the first measurement to be obtained. That pressure can be easily calculated. Thus, let the jet be turned so as to throw its column of fluid into the air alongside a meter scale; the distance of the upward throw of water is obviously the pressure of impact on the thumb in centimetres or millimetres of water. The jet is kept less than half an inch from the thumb, so that a small deduction from the scale reading can be allowed for that distance. A fairly accurate measurement is thus achieved of the pressure necessary to blanch a 2 to 2·5 mm. of skin area. The water should be kept at body-heat to prevent cooling of the vascular area.

When readings of pressure are taken at heart level by this method, they

do not materially differ from the readings of pressure obtained by V. Kries. But be it noted, as the flow is stopped in the blanched part the readings are of arteriole, not capillary pressure.

Moreover, there remains to be considered the resistance of the convex horny layer of skin to compression. There are also the resistance of the vascular wall to compression, the friction of the blood as it is expelled from the compressed area, the resistance of the lymph or tissue fluid surrounding the capillaries. In the frog's web these resistances are very small. One of us (L. H.) has shown that a pressure of 1 cm. of water applied to the membrane of the Roy and Graham Brown apparatus is sufficient to drive the corpuscles quickly along the vessels of the web wherein the circulation has been arrested. Up to the present no adequate method has been devised for estimating these factors and the resistance of the horny layer of the skin in man. Their sum can be accurately estimated provided one can reduce the blood-pressure in the compressed area to zero. Then the pressure taken to blanch the area is a measure of the resistance factors.

To reduce the blood-pressure to zero is not an insoluble problem. It is known that the veins of the dorsum of the hand at or below the heart level can be seen to bulge. The lower the hand is placed, the tenser the veins become. Now if the hand is raised above the heart level, at a certain point the veins collapse, because the venous pressure has been overtopped by gravity. The venous pressure in the hand is now zero. If the hand be raised still higher and the brachial artery occluded, the arterial pressure entering the hand is in its turn reduced to zero. Accordingly the capillary pressure becomes zero. A tourniquet of rubber tubing can be used or the cuff of the sphygmomanometer connected with a compressed air cylinder so as to secure a rapid application of pressure to the artery. The pulse is found to be absent in the radial artery, and if we blanch a large area of skin by compression the area remains white. This in itself is a proof of zero pressure. If an elastic area is compressed there is, of course, a recoil effect on removal of compression which tends to suck the blood back into the area. This is seen notably in the 2 to 2·5 mm. area compressed by the jet. The smaller the area compressed, the greater the recoil effect.

Had the blood in the area been endowed with a pressure, then the large blanched area would quickly blush once more. Further, in time the blood tends to pass by gravity or muscular movements to the interior of the thumb or into big superficial veins, which is in itself another proof of the absence of arteriole pressure in the area. The phenomenon of discoloration in dependent parts in the dying patient or in the dead is analogous. In order to get enough blood in the capillaries to give a clear index, it is advisable to put a rubber band round the wrist before elevating the arm and compressing the artery, and then to remove this band.

If the jet be projected on the thumb when the area has been thus brought to zero pressure, a measure of the pressure in centimetres or millimetres of water required to blanch the area is now obtained. This pressure measurement—the measurement of resistance factors—has to be subtracted from the original measurement obtained when the normal conditions of pressure pertained in the arterioles.

The diminished reading is now the actual arteriole pressure.

To cite examples:

Subject E.—Hand at heart level. Pressure to blanch 2·5 mm. area = 35 cm. water. Resistance measurement (after vascular area reduced to zero pressure) = 24 cm. water. Difference = arteriole pressure = 11 cm. water.

The readings for this subject on other occasions were: 45 and 31; difference = 14 cm. water. 41 and 31; difference = 10 cm. water.

Subject M.—The readings for subject M were 43 and 31; difference = 12 cm. On another occasion, 50 and 35; difference = 15 cm.

Subject W.—The readings for subject W were 40 and 26; difference = 14 cm. water.

The arteriole pressure at heart level comes out then as about 10 mm. Hg., and this is too high a reading because the method of compression is not momentary enough and allows time for banking up. The observations made by one of us (L. H.), using the Roy and Graham Brown method, show that the capillary pressure in the mouse's ear and mesentery is not more than 2–3 cm. of water—about 2 mm. Hg. The deductions, then, that have to be made from all the usual readings that are in vogue for capillary blood-pressure are important, and must be reckoned with in consideration of physical theories of lymph production.

The physical theory of the production of urine which postulates a glomerular capillary blood-pressure amounting to a figure as high as 20 per cent. below carotid blood-pressure also requires to be modified very materially. One of us (L. H.) has for many years insisted that the supposed filtration pressures do not and cannot exist because the tissue fluid pressure balances the capillary pressure. This is seen to be so in the case of the brain and eye. The acceptance of the low value of capillary pressure makes the production of capillary stasis in disease generally, or in peripheral parts, more intelligible. Thus the hands of a dying patient may show obvious signs of capillary stasis while the pulse is still quite palpable. But if the arteriole pressure be normally as low as 5 mm. Hg. any failure of the force of the heart-beat will, by sending the pressure lower, produce the condition of stasis. There is in the capillary field little margin of safety.

SUMMARY.

That pressure which stops the flow through and expresses blood from the skin has been generally taken as signifying the capillary blood-pressure. In truth it indicates the pressure in the small arteries which feed the compressed part after an allowance has been made for error arising from the pressure required to deform the convex horny layer of the skin. The method devised measures this error and arrives at the true pressure in the small arteries. The pressure of a small jet of water is found which will just blanch the skin of the hand held at heart level (1) in the normal condition, and (2) with the capillaries at zero pressure after the veins of the forearm have been emptied by gravity and the brachial artery occluded by a tourniquet. When (2) is deducted from (1) a reading of about 10 mm. Hg. is obtained. Reference is

made to previous measurements of capillary pressure in the brain, etc., and to recent experiments carried out on transparent membranes of frogs, mice, etc., by one of the authors (L. H.). These show that a momentary compression equal to 2–5 cm. water suffices momentarily to check the flow of corpuscles in the arterioles or its main capillary branches. The true capillary pressure as indicated by these figures is a very low one, and theories of lymph formation, etc., based on a capillary pressure of some 30 mm. Hg. must be modified accordingly. The relation of the swelling of the tissue cells to the capillary circulation in inflammation is discussed, also the relation of the pressure of the cerebro-spinal fluid, aqueous humour and salivary secretion.

REFERENCES.

1. Danzer, C. S., and Hooker, D. R.—"Determination of the Capillary Blood-pressure in Man with the Micro-capillary Tonometer," *Amer. J. Physiol.*, 1920, **52**, 136.
2. Hill, L.—'Further Advances in Physiology,' London, 1909 (Arnold), p. 149. "Blood-vessels and Pressure," *Lancet*, 1920, **1**, 359.
3. Roy, C. S., and Brown, J. G.—"The Blood-pressure and its Variations in the Arterioles, Capillaries and Smaller Veins." *J. Physiol.*, 1879-80, **2**, 323.
4. Hill, L.—"The Capillary Blood-pressure," *J. Physiol.*, 1920, **54**, xxiv.
5. Hill, L.—'The Physiology and Pathology of the Cerebral Circulation,' London, 1896 (Churchill). "The Experimental Pathology of the Cerebral Circulation," Allbutt and Rolleston's *System of Medicine*, London, 1910, **8**, 3 (Macmillan).
6. Hill, L., and Flack, M.—"The Secretion of the Aqueous and the Intra-ocular Pressure," *Proc. R. Soc.*, B., 1912, **85**, 439.
7. Hill, L., and Flack, M.—"The Salivary Secretion," *ibid.*, 1912, **85**, 312.

SPONTANEOUS AND ARTIFICIAL PULMONARY LESIONS IN GUINEA-PIGS, RABBITS AND MICE.

H. B. MAITLAND, M.B., MARY L. COWAN, B.A., M.B., AND H. K. DETWEILER, M.D.

From the Department of Pathology and Bacteriology, and the Department of Medicine in the University of Toronto.

Forwarded for publication October 25th, 1920.

INVESTIGATIONS made in searching for a filter-passing virus as a possible factor in the ætiology of epidemic influenza have been reported in a former paper.(1) No evidence was found to support such a possibility, but in the animals used for the experiments, particularly the guinea-pigs, two types of pulmonary lesions, hæmorrhagic and proliferative, were observed. It was shown that these lung changes did not indicate experimental influenza. They were believed not to be the result of the inoculation of Berkefeld filtrates of material from influenza patients—sterile in ordinary culture—which the animals received, because a similar percentage of lesions occurred in animals inoculated with control material, and also in untreated animals from the stock pens.

The hæmorrhage and the proliferation were distinctly different types of abnormality. Each was observed alone in many cases. When they occurred together the association was of no fundamental significance, neither being dependent on the other. Therefore they were considered to have different origins. In order to determine, if possible, how they were produced, the experiments to be reported in this paper were performed.

THE HÆMORRHAGIC TYPE OF LESION.

Apart from the papers relating to the ætiology of influenza, this striking pulmonary lesion does not appear to have received attention in the literature. Since this paper is specially concerned with it, a recapitulation of a former description (1) may be permitted. This lesion was most marked in guinea-pigs, and the following description refers to them.

On the surface of the lung the hæmorrhagic area had various forms. Sometimes the whole lung was thickly spotted with small hæmorrhages 2 to 3 mm. in diameter, and on section they were seen to be deep in the lung-tissue as well. In other instances solid hæmorrhagic lesions appeared on the surface in irregular-shaped areas, from 3 or 4 mm. to 8 or 10 mm. in diameter. These usually had a dark red centre and a brighter red periphery. Occasionally

the greater part of one lobe, or of several lobes, was irregularly involved. In rabbits the lesion had exactly the same appearance, but was less extensive. It occurred also in mice.

Microscopically the lesion was characterised by several peculiar features. There was extensive extravasation of erythrocytes into the alveoli and even into the bronchioles. In the most extreme degree the air-holding tissue in a focus of hæmorrhage was almost obliterated, and only a few empty alveoli could be seen, isolated from one another, in a mass of red cells. In places there was less lung-tissue involved, and the hæmorrhage was more diffuse, the type of change being essentially the same, differing only in extent. There was also hæmorrhage into the alveolar walls, and engorgement of the capillaries. The blood-cells were not deformed, and stained evenly and well. Hæmosiderin was not present. One of the most striking features was the absence of leucocytic reaction.

After it had been established that this lesion did not result from inoculation (1) it became apparent that, in general terms, the cause must be some agent acting during life—infection, protein poison, etc.—or, on the other hand, some agonal phenomenon due possibly to the method of killing which had been adopted in the experiments. Against the view that the hæmorrhages were present in the guinea-pigs during life there were several arguments. The animals in which hæmorrhagic lungs were found—even the most extensive—did not appear to be ill. Their temperature was normal. There was no free fluid, or fibrin in the pleural sac. The blood cells were not degenerated. If they had been outside the vessels for any considerable time prior to death one would have expected to see some signs of their disintegration, and especially to have found some hæmosiderin. Neither was observed. Further, although foci of hæmorrhage may be a frequent result of infection, or of inoculation with pathogenic micro-organisms, in such instances one expects to find an associated local leucocytic reaction. This was entirely absent in the lesion under discussion, and the fact was so remarkable that in conjunction with the other features that have just been mentioned it seemed very improbable that the hæmorrhage could have resulted from an infection. The correctness of this deduction was confirmed by the results of culturing the lungs. The aërobic cultures in glucose ascitic broth made from about 150 hæmorrhagic guinea-pig lungs were almost always sterile. The few exceptions formed such a small percentage of the total that they did not detract from the value of this evidence.

The other alternative, then, was to consider the method of killing and the mode of death.

The routine method had been to hit the guinea-pig a blow on the back of the head. It became unconscious immediately. There was usually some tonic contraction of the muscles and a few gasping respirations before movement ceased and complete relaxation obtained. For the succeeding five minutes or more, during preparation for the autopsy, the heart continued to beat, making a quite forcible impulse on the chest-wall. Cessation of all functions was therefore neither immediate nor simultaneous.

It was thought that the only way in which the question of the presence or absence of these hæmorrhagic lesions during life could be definitely settled

would be actually *to see* the lungs before any changes could possibly take place as the result of killing the animal. For this reason the following experiment was made in the hope of obtaining the lungs unaltered by any agonal phenomena which might have occurred in our previous work.

Experiment I.—Sixteen guinea-pigs were killed by cutting through the anterior thoracic wall and into the heart with one snip of the scissors. The chest was then immediately opened more widely and the diaphragm incised. Thus respiratory movements had no effect on the lungs, the circulation through them was destroyed, and the possibility of agonal lesions was reduced to a minimum. The lungs were removed and examined without delay.

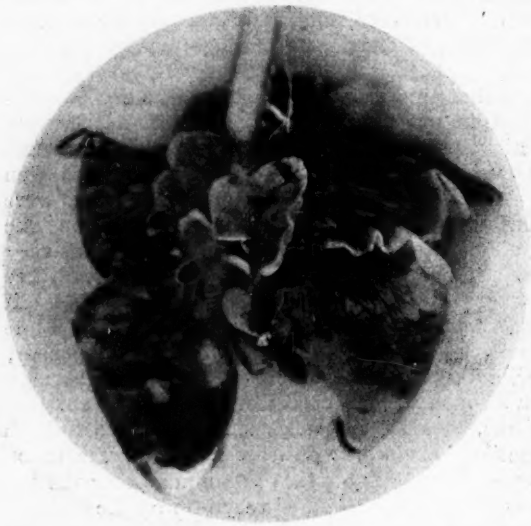

FIG. 1.—Lung of guinea-pig (*Experiment II*), killed by a blow on the head. Areas of hæmorrhage are present on two large lobes and one small lobe.

All of these lungs were free from the hæmorrhagic lesion. In two there were, however, two or three superficial bright red flecks of hæmorrhage, situated on different lobes. They were about 2 to 3 mm. in diameter, and were so evidently recent, so isolated and small that no one could possibly mistake them for experimentally produced disease. Apart from these the gross appearance was quite normal.

Two other guinea-pigs were treated in a similar way except that the cut which opened the thorax did not penetrate the heart, so that this was not opened until about one minute later. The lungs from each showed hæmorrhages. The first one had several areas, 6 to 8 mm. in diameter, involving parts of two lobes. The colour on removal was bright red, but in a few minutes the central part became much darker. On section it extended solidly into the lung—in other words it was a typical hæmorrhagic lesion. The second lung was similar.

Experiment II.—In order to compare accurately the effects of the different methods of killing, seventeen pigs from the same pen, raised under the same conditions, were killed by a sharp blow on the back of the head. This was the method used as a routine in the influenza experiments.

After the animal had been struck respirations sometimes ceased immediately, but usually continued for one or two minutes. The heart beat forcibly for five to eight minutes longer and a marked impulse could be felt on the chest-wall. When it became very faint the thorax was opened and the

lungs removed. This length of time corresponded to the time occupied in sterilising the skin and preparing for the autopsy in the former experiments.

Six of these lungs had typical hæmorrhages (Fig. 1), the lesions corresponding in every way to those found during the influenza investigations. The result contrasts markedly with that of *Experiment I*. The only way to account for the presence of the hæmorrhages in the second experiment and their absence in the first was to assign their cause to the difference in the terminal state of the guinea-pigs. In the first experiment respiration and circulation ceased together. In the second the heart continued to beat forcibly for about five minutes after respiration had stopped. The guinea-pigs used for the two experiments were similar. Some were killed alternately, first by one method and then by the other; some were killed by one method or the other in batches on successive days.

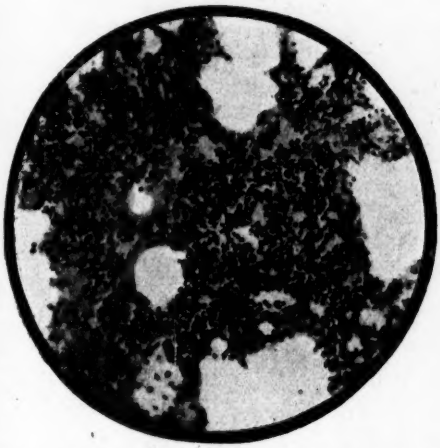

FIG. 2.—Section of lung in *Fig.* 1, showing area of hæmorrhage of moderate extent. (× 80.)

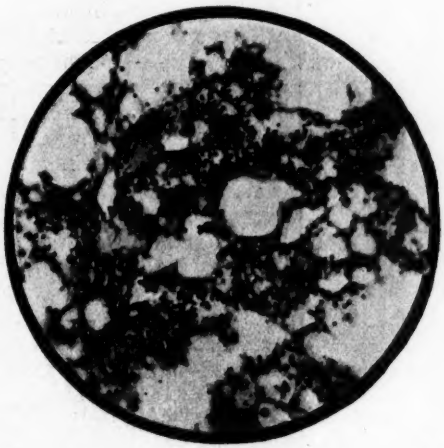

FIG. 3.—Section of lung in *Fig.* 1, showing a small amount of diffuse hæmorrhage. (× 80).

The results of *Experiment I* corroborated the evidence previously discussed, supporting the view that the hæmorrhages were not present in the lungs during life, and showed that by employing a suitable method of killing they could be avoided. They were, therefore, an agonal phenomenon. Sixteen is a comparatively small series, but in no other series of equal size examined by us have hæmorrhages failed to appear, and therefore this conclusion seems justifiable.

The practical importance of this lesion is, of course, the relation it has to the experimental pathology of the guinea-pig's lung. In some circumstances the method of killing used in *Experiment I* might be a satisfactory procedure, but it has the disadvantage of preventing the making of cultures from the thorax, and since this is often important, a third method was tried in order to overcome this difficulty.

Experiment III.—Twelve guinea-pigs from the same pen as the others were killed by rapid chloroform anæsthesia. The chloroform was poured on absorbent cotton in the bottom of a beaker in which the animal's head and shoulders were placed. Respiration ceased in from $1\frac{1}{4}$ to $3\frac{3}{4}$ minutes. The force of the cardiac impulse rapidly weakened during that time, and when breathing stopped it was barely perceptible, and sometimes could not be felt. The thorax was then opened immediately.

Ten lungs were free from typical hæmorrhage but one of them had some small isolated flecks.

FIG. 4.—Lung of guinea-pig (*Experiment III*), killed by rapid chloroform anæsthesia. Diffuse distribution of small areas of hæmorrhage, some of which coalesce to form larger lesions.

One lung was evidently diseased. About half of one lobe was shrunken, smooth, firm and dull salmon-red in colour. Similar tissue was scattered throughout in very small patches, giving the surface a speckled appearance.

The twelfth lung was riddled with hæmorrhages from 2 to 6 mm. in diameter, some coalescing and forming irregular blotches on the surface—an extensive typical lesion (Fig. 4).

Thus killing by chloroform does not entirely obviate this type of lesion, although it gives fewer hæmorrhagic lungs than a blow on the back of the head and therefore is to be preferred to that method. Bleeding by cutting the vessels in the neck has been tried (1) but failed to produce the desired result.

Three pigs out of twenty-three killed in this way had hæmorrhagic lungs. The method of *Experiment I* appears to be successful in avoiding hæmorrhages, but because it interferes with bacteriological examination of the organs it very frequently cannot be employed.

Although guinea-pigs were used for the elucidation of this pulmonary lesion, the same type of hæmorrhage was also observed in rabbits and mice which had been inoculated, along with the guinea-pigs, with filtrates of influenzal material during the experiments on influenza. It therefore seems reasonable to believe that the similar lesions in them were caused in the same way as in guinea-pigs and were produced at the time of death. It is necessary to take into account the possibility of these pulmonary hæmorrhages when interpreting *post-mortem* findings in laboratory animals, in the same way as allowance should be made for the proliferative lung lesions. The chief features by which they may be distinguished are the absence of leucocytic reaction in the tissues and the absence of hæmosiderin.

FIG. 5.—Area of hæmorrhage from lung in *Fig.* 4. (× 160.)

THE PROLIFERATIVE LESION.

The liability of the guinea-pig to spontaneous infections is well known and excellent papers on this subject are available (3) (4). The stock of most laboratories suffers occasionally from epizootics, and in the absence of epidemics the animals may harbour pathogenic bacteria, which do not cause serious infections on account of the immunity which the animals appear to have developed in these circumstances. Theobald Smith (2) points this out in discussing the pneumonia due to *B. bronchisepticus*, and mentions that stock which are carriers of this organism are not suitable subjects for experiments regarding its pathogenicity.

Although the animals used for the influenza experiments appeared to be healthy and the lungs were usually sterile, *B. bronchisepticus* was isolated from the lungs of sixteen guinea-pigs. Five of these showed no gross lesions. The lungs of the remaining eleven (six of which had small hæmorrhages) presented a lesion which in the gross appeared as an irregular-shaped part of one lobe depressed below the level of the normal lung surface. It was a reddish colour, smooth, firm and homogeneous, and extended in a solid, sharply outlined mass from one surface of the lung to the other. Microscopic examination showed that this was a condition of atelectasis. There was no inflammatory reaction. This change was also found in lungs that did not yield any bacteria on culture. Therefore we are not able to associate *B. bronchisepticus* with any constant

lung abnormality in this series of guinea-pigs, nor are we able to ascribe to it any ætiological significance deduced from experimental data.

The histological character of the proliferative lesion, which has been described in the former paper (1), showed it to be a slow inflammatory reaction, and it may well be, when one considers the pathogenic properties of the organism, that *B. bronchisepticus* was the ætiological agent, although we cannot go farther than to suggest this possibility.

The microscopical appearance (Fig. 6) of the lesion was as follows: The alveolar walls were thickened, due to proliferation of endothelial cells. Where the change was least the outline of the alveoli could easily be traced, but the air-space of the lung was considerably lessened. In places of greater change single alveoli were completely obliterated by the proliferation; others nearby were only a small fraction of their original size, and the outline of the alveolar walls was lost in the diffuse mass of cells. Where this was most marked the tissue was solid and the air-space entirely obliterated. The cells were rather large and irregular in outline, the nuclei were large, central, oval or round, and stained with moderate intensity. The cell masses included capillaries. Giant cells were occasionally seen. A recent thrombosis of a small branch of a pulmonary artery was associated with this type of lesion.

FIG. 6.—Section of the lung of an untreated guinea-pig showing the proliferative change. (× 120.) This section shows proliferation of moderate extent. In other areas the cells were continuous, obliterating all the air-space; the same change also occurred in small patches and was less extensive.

The sixteen strains of *B. bronchisepticus* were identical morphologically and in their cultural appearances, and corresponded to the description of the organism given by McGowan (2), Theobald Smith (3), and Holman.(4) It was a Gram-negative, motile bacillus showing marked pleomorphism. It stained easily and very often unevenly, the ends being more heavily stained than the centre. When first isolated it grew luxuriantly in plain broth or on plain agar in forty-eight hours at 37·5° C., but after several sub-cultures an equal growth appeared after twenty-four hours in the incubator or after forty-eight hours at room temperature.

On agar the colonies varied in size from 1 to 4 mm. in diameter, were discrete, round, moist, greyish-white and translucent. In broth the first cloudiness appeared at the surface. Later the whole became cloudy, a pellicle developed, and a tenacious, white stringy deposit formed in the bottom of the tube.

From agar cultures the organisms tended to be smaller, 0·5 × 1·0 μ, and showed less variation in size and staining. From broth the pleomorphism

was marked; filamentous forms and short chains were seen and the organisms were somewhat larger.

Gelatin was not liquefied. Litmus milk was not changed at the end of a week. On potato there was a luxuriant growth, and in from three to seven days it became a deep yellowish-brown colour. Endo's medium was not fermented, likewise neither glucose, lævulose, maltose, mannite, saccharose, galactose, raffinose, salicin, dulcite, dextrin nor inulin.

SUMMARY AND CONCLUSIONS.

A pulmonary lesion in guinea-pigs has been described which was characterised by hæmorrhage into the alveoli and sometimes into the bronchioles. There was no leucocytic reaction in the lung-tissue, and no hæmosiderin was present in the cells of the lung. This lesion is an agonal phenomenon. The same lesion occurred in rabbits and in mice, and is considered in them to be also an agonal phenomenon. Killing by rapid chloroform anæsthesia produced fewer agonal lesions than a blow on the back of the head. A method of killing has been described by which hæmorrhage was not produced.

A second type of lesion has been noted which is essentially a slow proliferation of endothelial cells. It is a spontaneous animal disease whose origin is undetermined.

The hæmorrhagic and proliferative lesion frequently occurred in the same area, but neither was dependent on the other.

B. bronchisepticus was isolated from the lungs of sixteen guinea-pigs, but it was not possible to associate its presence with the proliferative lesion.

REFERENCES.

1. Maitland, H. B., Cowan, Mary L., and Detweiler, H. K.—"The Ætiology of Epidemic Influenza: Experiments in Search of a Filter-passing Virus," *Brit. J. Exper. Pathol.*, 1920, **1**, 263.
2. McGowan, J. P.—"Some Observations on an Epidemic of 'Distemper' among Laboratory Animals," *J. Pathol. and Bacteriol.*, 1911, **15**, 372.
3. Smith, T.—"Some Bacteriological and Environmental Factors in the Pneumonias of Lower Animals, with Special Reference to the Guinea-pig," *J. Med. Res.*, 1913–14, **29**, 291.
4. Holman, W. L.—"Spontaneous Infection in the Guinea-pig," *J. Med. Res.*, 1916–17, **35**, 151.

THE NATURE OF THE EFFECT OF BLOOD-PIGMENT UPON THE GROWTH OF *B. INFLUENZÆ*.*

PAUL FILDES.

From the Bacteriological Laboratory of the London Hospital.

Received for publication November 1st, 1920.

DURING the preparation of peptic digests of blood for the growth of *B. influenzæ* by a technique already described (1), it was found that sometimes a separation of the blood-pigment from the other blood constituents took place. The blood-pigment deposited as a fine dark brown powder, leaving a clear yellow supernatant fluid. If such a mixture was filtered through paper, the precipitate could be gathered, washed with distilled water, redissolved in caustic soda and adjusted to a reaction of P_H 7·6.

The brown precipitate consisted of hæmin, the hydrochloride of hæmatin, and upon being washed and dissolved in soda was converted into alkali hæmatin. The filtrate showed no spectrum of blood-pigment and a "peroxydase" reaction with tincture of guaiacum and ozonic ether only in very low dilutions. Thus it contained extremely small quantities of blood-pigment.

If this alkali hæmatin was added to agar in large or small quantities no growth of *B. influenzæ* was obtained. Similarly the filtrate up to 25 per cent. failed to promote growth, but when small quantities of the hæmatin and filtrate were added together, then a copious growth resulted.

From such an experiment it follows that blood-pigment alone when combined with ordinary nutrient agar is incapable of supporting the growth of *B. influenzæ*, and that a substance present in the non-pigmented fraction of blood is also essential.

By repeating the above experiment with washed red corpuscles instead of with defibrinated blood, it is shown that the two essential factors are still present, apparently in undiminished quantity, and thus it may be assumed that the non-pigmented fraction is derived from the formed elements of the blood.

The object of the present work was to throw light upon the part taken by the pigment in the growth of the bacillus.

It is known that the bloods of different animals behave in different ways towards *B. influenzæ*, and on this account the experiments have been confined to the blood of one animal, namely the sheep. Further, it is known that very

* Work carried out under grants from the Medical Research Council.

small quantities of blood-pigment suffice for growth, and therefore in test cultures it is necessary to transfer only minimal quantities of diluted bacteria from a medium poor in pigment.

Six different strains of *B. influenzæ* have been used, and no differences in behaviour have been observed.

THE RELATION OF BLOOD-PIGMENT TO THE GROWTH OF *B. INFLUENZÆ*.

In accordance with the original description of Pfeiffer [2] no growth of *B. influenzæ* has ever been obtained in the absence of blood-pigment.

It will be recalled that the opposite was claimed by Cantani [3], but refuted by Ghon and von Preyss [4], Luerssen [5] and others. The mistake of Cantani was due to his use of the spectroscope as a test for blood-pigment; but it has been shown that quantities of pigment are sufficient which cannot be detected by this test. For instance, the medium of Levinthal is quite colourless and gives no absorption spectrum, but the guaiacum reaction is always positive. The statement is also sometimes met with that *B. influenzæ* can grow without blood-pigment in symbiosis with *Staphylococcus aureus* and other bacteria, but such growths have not been observed in the present work if care is taken to transfer no blood-pigment with the inoculum and if no pigment is present in the medium (guaiac test). It is, however, clear that very small quantitie of blood-pigment suffice for growth.

THE QUANTITATIVE RELATION OF BLOOD-PIGMENT TO THE GROWTH OF *B. INFLUENZÆ*.

It has been stated by Davis [6], and his statement has been generally accepted, that 1 in 180,000 of human hæmoglobin is sufficient to allow growth of *B. influenzæ*, but such a high dilution has not been confirmed in the present work with sheep's blood. Davis obtained his results by diluting human blood with distilled water and adding this to agar. He then found the minimum quantity which would allow growth and calculated the amount of hæmoglobin in this quantity. When subcultures were made in series upon the higher dilutions it was found that they were apt to die out.

When repeating these experiments pure alkali hæmatin was used, and added in falling quantities to agar made up with an excess of the colourless filtrate of digested corpuscles. When these tubes were inoculated with *B. influenzæ* growth occurred up to a dilution of 1 in 5 million of hæmatin. The experiments were, however, repeated. A fresh pipette was taken to make each dilution of hæmatin, and the bacteria used for the inoculation were washed once by suspension in saline before being transferred. In these experiments it was found that 1 in 50–63,000 of hæmatin was the limit in four separate series of tests. It may be taken that there is 0·8 per cent. of hæmatin in whole blood, and therefore the necessary dilution of sheep's blood is 1 in 400–500.

It is probable that growth at higher dilutions depends upon error in carrying over blood-pigment from tube to tube or with the bacteria from the original medium, and the fact that Davis's cultures were apt to die out on subcultivation on the higher dilutions supports this view.

THE BLOOD-PIGMENTS WHICH ARE NECESSARY FOR THE GROWTH OF *B. INFLUENZÆ*.

According to Pfeiffer hæmoglobin is essential for the growth of *B. influenzæ*, but it was soon shown that this was not the case. Grassberger (7) and Ghon and Preyss both found that hæmatin would allow growth in symbiosis with *Staphylococcus aureus* but not otherwise. Similarly Voges showed that heated blood containing no hæmoglobin but hæmatin was a good medium, but both Grassberger and Ghon and Preyss pointed out that the formation of hæmatin could not be the factor, because hæmatin, whether heated or not, did not support growth. In a later paper, however, Ghon and Preyss (1904) (4) found that hæmatin would allow growth without symbiosis if a little sterile milk was smeared upon the surface of the medium.

Looking at these original experiments with later knowledge, it is clear that the failure to obtain growths with hæmatin was due to the absence of the essential body which is contained in the colourless fraction of blood-corpuscles. This factor, it may be presumed, is supplied in a slight degree by milk or by the action of *Staphylococcus aureus*.

Nearly all early writers tested the effect of other blood-pigments without success.

In the present work it was not possible to obtain pure hæmoglobin from the sheep, and therefore the experiments were carried out with admittedly impure samples prepared as follows: 100 c.c. of washed deposited sheep's corpuscles were placed in a stoppered bottle with an equal volume of salt solution and 40 c.c. of ether. The mixture was thoroughly shaken, allowed to stand on ice overnight, and the hæmoglobin was then removed from the supernatant stroma. By warming and shaking most of the ether was removed, and the solution was then filtered through a Berkefeld filter.

Pure specimens of sheep's hæmatin were prepared by two methods—by alcoholic extraction of a dry mixture of anhydrous potassium carbonate and defibrinated blood, and also by the peptic digestion of sheep's corpuscles and the separation of hæmin. In each case the pure dry product was dissolved in water or dilute soda solution with subsequent neutralisation, in a strength of 2 per cent.

A specimen of impure hæmatoporphyrin was obtained from Dr. Laidlaw. Upon analysis by Mr. Candy this was found to contain small quantities of iron—whether in the form of hæmatin or not was not determined.

Attempts to use hæmochromogen in a culture of *B. influenzæ* were abortive. Firstly, it was not possible to reduce hæmatin with the feeble reducers and at the slight degree of alkalinity permissible in a culture medium. Secondly, as will appear later, the presence of any reducing substance, even so feeble a substance as glucose, inhibits or seriously interferes with the growth of *B. influenzæ*.

All blood-pigment solutions were sterilised and preserved by means of chloroform according to a technique previously described.(8)

When testing these preparations they were mixed with ordinary nutrient agar in such quantities as would bring the concentration of pigment in the medium to the equivalent of 1 in 80 of whole blood. It is not necessary to remove the chloroform except when a liquid medium is being used.

The results of the tests were as follows:

Hæmoglobin.—The results with hæmoglobin alone in agar were somewhat variable. Sometimes no growth was observed, but at others it was possible to detect a number of extremely fine colonies in twenty-four hours. When mixed with the colourless filtrate the growth was more constant and pronounced, but even then not invariable and when present always small.

It will be understood that the determination of the presence or absence of growth is a relatively gross matter. This is particularly the case with *B. influenzæ* when grown upon blood-agar made from human or some other bloods. In twenty-four hours the growth is often so excessively fine as almost to escape observation, especially when the blood has been laked before mixing with the agar. In the present paper by growth is meant not the type of growth which is obtained on Pfeiffer's blood-smeared agar, but the copious growth, like that of a young typhoid culture, which is obtained upon heated blood, peptic blood agar (1), or good specimens of Levinthal's medium. On the other hand, by no growth is meant that no colonies could be seen on the medium with a hand-lens after forty-eight hours.

When hæmoglobin is mixed with a medium and incubated the pigment rapidly changes to methæmoglobin, and therefore the use of the term "hæmoglobin" is inaccurate. There is some reason to suppose that this change is responsible for some of the variation observed in the growth obtained. In old specimens of hæmoglobin, largely converted into methæmoglobin, the growth tends to be more obvious than when the pigment is fresh, and it is probable that were it possible to maintain the pigment as hæmoglobin no growth would occur.

Hæmatin.—As already indicated, hæmatin mixed with agar never grew *B. influenzæ*, but copious growths were obtained when the colourless fraction of a peptic digest was added.

Hæmatoporphyrin.—This substance failed to grow the bacillus either alone or when mixed with the colourless fraction.

From these experiments it was concluded that an iron-containing pigment was necessary for the growth of *B. influenzæ*, and that the protein-containing pigment was very much less effective than that free from protein.

The necessity for iron was recognised by Pfeiffer and later workers, and they had attempted to reintroduce it in the form of various organic and inorganic preparations without success. I have tested the effect of ferric ammonium citrate, dialysed iron, colloidal iron, metallic iron, and inorganic ferric and ferrous salts both alone and with the colourless filtrate or with the filtrate and hæmatoporphyrin without obtaining growth of *B. influenzæ*, but such results need merely indicate that the experimentally-introduced iron is not in the same state or under the same conditions as the iron of hæmatin.

THE OXYGEN REQUIREMENTS OF *B. INFLUENZÆ*.

The fact that blood-pigments are associated with the function of oxygen-carrying, and that iron salts are believed to act as catalysts to accelerate the oxidation of materials in the life of cells, led to experiments being carried out upon the relation of *B. influenzæ* to oxygen.

It is known that *B. influenzæ* is a strong aërobe, according to some ([9]) an obligate aërobe, and it is stated not to grow under anaërobic conditions. Further, it is known that good growths in liquid media are only to be obtained when a large surface of the medium is exposed to the air. The growth takes place almost entirely in the superficial layers of the culture, and therefore the relation of the aërated surface to the bulk of the medium has a great bearing upon the thickness of the growth when the culture is shaken up and dispersed. A liquid medium, 2 or 3 mm. deep, in a flask will grow very much more copiously than the same bulk of medium in a tube. A copious growth is thus dependent upon a rich supply of oxygen.

If, however, slopes of medium containing peptic digest of blood-corpuscles are inoculated with *B. influenzæ* and incubated under anaërobic conditions (McIntosh and Fildes' anaërobic jar ([10])), a growth of minute colonies will still be found in twenty-four hours—more distinct, in fact, than an aërobic growth on Pfeiffer's blood-smeared agar. If, after two or three days' incubation, this culture is subcultured and this subculture incubated aërobically no growth will occur, and it is thus clear that under such anaërobic conditions *B. influenzæ* will grow normally up to a certain point and will then die out. From this experiment it follows either that the anaërobic apparatus in use had not extracted all of the atmospheric oxygen and that a remnant had been left to allow this growth, or that there is an oxygen store in the medium which is not capable of being extracted by a simple process of diffusion.

The method of oxygen absorption with spongy palladium in an atmosphere of hydrogen is well known to be suitable for the surface growth of strict anaërobes, and therefore it is improbable that sufficient atmospheric oxygen would be left to allow such a considerable growth of a strong aërobe. The following experiment indicates that the oxygen is not obtained from the atmosphere, but from the medium :

Two small plates containing a very thin layer of agar medium *plus* the peptic digest were placed in a McIntosh and Fildes' anaërobic jar. A magnetic apparatus was also included, by which a small drop of a suspension of *B. influenzæ* in broth could be dropped upon the plate from a pipette without opening the jar. A drop of this suspension having been placed upon plate 1, the apparatus was shut, the oxygen absorbed and the jar incubated for two days. A drop of the suspension was then placed upon plate 2 and after a further incubation of two days the jar was opened. On each plate was an equal, very feeble growth of *B. influenzæ*. These were subcultured, and the bacteria on plate 1 were shown to be dead but those on plate 2 living. Thus, whatever oxygen the atmosphere of the jar contained at the beginning of the experiment, there was not enough left to support growth continuously on plate 1; nevertheless, plate 2 grew equally well under these conditions and therefore must have derived its oxygen from the medium, although the bulk of this was small and the area for diffusion great.

If experiments are made under conditions which would tend to deprive the medium still more of dissolved or loosely combined oxygen, namely, if the jar is repeatedly exhausted and flooded with hydrogen, then the growth of *B. influenzæ* becomes still less and may even become inappreciable.

The dependence of *B. influenzæ* upon a store of oxygen in the medium is

also shown by its behaviour in shake cultures. Deep agar tubes containing the peptic digest were placed in boiling water until similar tubes containing methylene-blue in addition were completely reduced. The deep tubes were then rapidly cooled, inoculated with *B. influenzæ* upon a fine glass rod and set in cold water. A fine glass rod was found to be less liable to introduce air than a pipette. The zone of anaërobic conditions in these inoculated tubes could be judged by comparison with the methylene-blue tubes similarly treated, but with an uninfected rod.

As would be expected with an aërobic organism, growth occurred in the first twenty-four hours upon and just under the surface, but later growths appeared below the level of aërobic conditions and extended right to the bottom of the tube, where there was a complete absence of active oxygen. In other experiments it was shown that growth also took place actually in the presence of reduced methylene-blue, but the results were less constant.

If glucose is added to these tubes a result is obtained comparable to the pumping and flushing of the jar. The store of oxygen becomes less available and the growth is less marked and may be absent.

It is probable that the inconstant results of growth in the presence of reduced methylene-line blue is due to the blue acting similarly as a reducing agent. It may therefore be assumed that there is in the medium a store of oxygen upon which the bacteria may draw until it is used up, and that then they die. Under aërobic conditions the store is replenished from the atmosphere. When a reducing substance such as glucose is present in sufficient quantity, the oxygen store may be largely used up and the bacteria deprived of it. The growth under conditions which would remove merely dissolved oxygen, namely, pumping in the case of a thin layer of medium or boiling in the case of a shake culture, and the fact that growth will take place actually in the presence of colourless methylene-blue, indicate that the oxygen store is not merely dissolved in the medium, but is inactive in a state of loose combination with some substance in it.

It may be supposed that the medium contains an easily oxidisable and reducible substance—a "peroxide"—which is the direct source of oxygen to the influenza bacillus, and is immediately reoxidised in the presence of air. Such "peroxides" are familiar in connection with theories relating to the biology of cells in the body, and the activity of these substances is thought to be accelerated by the catalytic action of iron. Thus, it appeared possible that the iron of hæmatin might act towards this hypothetical "peroxide" or store of fixed oxygen in the medium as a catalyst, in order to accelerate the reduction of the peroxide by the bacterium.

THE FUNCTION OF THE HÆMATIN.

Pfeiffer himself thought that hæmoglobin might act as an oxygen carrier to the bacteria, but discarded the theory on the ground that carboxyhæmoglobin, which could not be an oxygen carrier, could nevertheless permit the growth of *B. influenzæ*. Pfeiffer was looking upon the oxygen function of the blood-pigment as similar to the function exerted in nature, but experiments with carboxyhæmoglobin and the fact also that hæmatin is more effective make this view improbable.

From the present work the function of the hæmatin might be considered to be two-fold: first, it might act as an iron salt as a catalyst, and secondly, it might act as a "peroxide," being a reducible substance easily re-oxidised in air.

There are some observations which oppose this second possible function and others which favour it. Firstly, it might be supposed that if hæmatin functioned as a peroxide, a shake culture of *B. influenzæ*, after several days' growth should reduce hæmatin in the deeper parts of the tube, and show the spectrum of reduced hæmatin, namely of hæmochromogen. This spectrum is not, however, observed; but, in fact, it cannot be observed, since it requires a greater concentration of alkali than is permissible in a culture of *B. influenzæ*. Thus, if a drop of N/1 NaOH is placed upon the surface of a deep agar culture of *B. influenzæ*, the spectrum of hæmochromogen spreads down into the depth of the tube with the alkali, and if the tube be cut at the spreading edge and the surface tested with thymolphthalein the reaction will be found to be over P_H 9·0. At this reaction *B. influenzæ* will not grow. This effect of alkali is not, however, dependent upon the growth of *B. influenzæ*, because the same result may be observed in an uninoculated tube, in which the mere reducing power of the medium has introduced anaërobic conditions. On the other hand, there is some reason to suppose that hæmatin may be reduced without the production of hæmochromogen. Bertin-Sans and Moitessier [11], for instance, describe a reduced hæmatin which does not give the spectrum of hæmochromogen, and further, the same authors note the existence of an oxidised product of hæmochromogen which differs from hæmatin in giving the bands of oxyhæmoglobin. In this connection it is of interest to remark that the bands of oxyhæmoglobin appear sometimes, under conditions undetermined, when the soda is added to the culture, as described above. Thus the absence of hæmochromogen bands from the culture does not necessarily imply that the hæmatin has not been reduced, since it is demonstrated that other reduced compounds than hæmochromogen exist. If, however, hæmatin functioned as a peroxide, the growth of *B. influenzæ* under anaërobic conditions should be proportional to the amount of hæmatin in the medium, but in practice this is not the case. If sufficient hæmatin is present to allow a meagre growth, this cannot be increased by adding much larger quantities.

The evidence is therefore, on the whole, against the probability that hæmatin functions as a peroxide in the medium.

The view of a catalytic effect of the hæmatin is supported by a certain amount of indirect evidence. It will be remembered that a catalytic action of blood-pigment is familiar in the misnamed "peroxidase reaction" of blood with tincture of guaiacum and ozonic ether. Ozonic ether, a peroxide, is alone practically incapable of oxidising the guaiaconic acid of the tincture, but upon the addition of a trace of blood the reaction is enormously accelerated, and the oxidisation takes place immediately with the formation of a blue colour. As ordinarily carried out with blood this reaction depends upon the added peroxide, but it is found that hæmatin is capable of oxidising the guaiacum alone without added peroxide, apparently by utilising dissolved oxygen or oxidised components of the resin.

These tests were carried out under conditions which simulated those

under which *B. influenzæ* grows. The various pigment solutions were diluted with nutrient broth to a dilution representing 1 in 32 of blood. The reaction was then adjusted exactly to P_H 7·2 (cresol-red). 5 c.c. of these dilutions were added to 5 c.c. of tincture of guaiacum diluted with distilled water, and the whole warmed for a minute or two and then left at room temperature.

Under these conditions laked blood, laked blood-corpuscles or fresh oxyhæmoglobin solutions gave no blue colour. Preserved hæmoglobin solutions containing methæmoglobin (these usually reacted acid to methyl-red) gave a slight blue colour which tended to disappear on standing. Peptic digest of blood gave rapidly a strong blue colour. Hæmatin gave a slowly developing but strong blue colour, and hæmatoporphyrin (containing, as mentioned, iron) a varying intensity of blue.

Thus there is a marked parallelism between this reaction and the growth of *B. influenzæ*. In the presence of oxyhæmoglobin little or no growth occurs and this reaction is negative. With old hæmoglobin preparations usually a feeble growth occurs and a feeble guaiacum reaction. The peptic digest or hæmatin permits a profuse growth and gives a strong reaction. Hæmatoporphyrin gives no growth, but the reaction is no doubt due to remnants of iron. Pure hæmatoporphyrin is said to give no guaiacum reaction even with ozonic ether.

The comparison may be carried even further in a striking manner. If to a medium containing peptic digest of blood and capable of producing a copious growth of *B. influenzæ* fresh laked blood, laked corpuscles or fresh hæmoglobin solutions are added, the growth is almost entirely or entirely inhibited. Similarly, the peptic digest *plus* fresh blood, etc., will give no guaiac reaction—in fact, a strong blue colour which has developed with peptic digest alone may be discharged by the addition of fresh hæmoglobin. Again, pigeon's blood is the only blood which has been found in the present work to give a profuse growth of *B. influenzæ* in its fresh state, and it is the only one which has been found to give a strong guaiac reaction.

From these experiments, therefore, although the evidence is indirect, it appears that the relation of blood-pigment to the growth of *B. influenzæ* is the same as the relation of the pigment to the oxidisation of guaiaconic acid; in fact, that the pigment acts as a catalyst to accelerate the transfer of oxygen from a peroxide in the medium to the bacillus.

THE NATURE OF THE DIFFERENCE BETWEEN HÆMATIN AND HÆMOGLOBIN AS MEDIA.

The present experiments throw some light upon the well-known phenomenon of the feeble or absent growth of *B. influenzæ* on "unchanged" blood, and the copious growth on blood "changed" by heat, digestion, or other methods. In the changed bloods the pigment is in the form of hæmatin and in the unchanged in the form of mixtures of hæmoglobin and methæmoglobin. In the former case the pigment has a marked catalytic action, but in the latter, except in the case of pigeon's blood, there is little or no catalytic effect demonstrable by the technique in use. This absence of effect may be due to some character of the structure of hæmoglobin rendering it feebly catalytic, or it may be due to an inhibitory action. There is no reason to

suppose that the catalytic action of hæmoglobin is inherently feeble, since it rapidly causes an oxidisation of guaiac upon the addition of ozonic ether. There is also direct evidence of an active reducing effect upon guaiac rendered blue by the action of hæmatin, or even guaiac rendered blue by the action of milk.

It is possible that, owing to its affinity for oxygen, hæmoglobin may take up any oxygen which becomes available by its catalytic action and thus deviate it from the bacillus, in the same way as glucose prevents growth and prevents the development of the guaiac reaction. This explanation might account for the greater growth which is obtained with old specimens, in which much of the hæmoglobin is changed to methæmoglobin before even the culture is incubated. On the other hand, experiments with carboxyhæmoglobin are not sharply confirmative. If a fresh hæmoglobin solution is placed in a thin layer in a flask and exhausted with the pump, pure carbon monoxide may be admitted and shaken with the pigment. The carboxyhæmoglobin so obtained may be incorporated in a medium and tested. The growth with carboxyhæmoglobin is practically the same as with hæmoglobin and so is the catalytic action, and yet this pigment has no affinity for oxygen. Spectroscopic examination, however, shows that carboxyhæmoglobin also is not stable in a medium. Indeed, it would be expected that in the course of incubation some would be reconverted on the surface and thus an inhibitory factor would be again introduced.

On the whole it appears probable that the feebleness of the growth upon an unchanged blood medium is due, at any rate in part, to the inhibitory action of hæmoglobin, this inhibition resulting from the intercepting of oxygen liberated from a peroxide by the catalytic action of iron in the pigment. Any process, such as slight heating or digestion or the addition of acid or alkali, which destroys the oxygen avidity of the pigment, at once allows the full catalytic action to operate and a copious growth occurs. According to this view such feeble growth as does occur upon unchanged blood media is due to the inevitable change to methæmoglobin and therefore a reduction in the inhibitory factor.

CONCLUSIONS.

(1) Growth of *B. influenzæ* has not been observed to occur in the total absence of blood-pigment.

(2) The quantity of blood-pigment necessary is small, but larger than has been stated.

(3) Probably oxyhæmoglobin and carboxyhæmoglobin are incapable of allowing growth, the feeble multiplication in these pigments being due to a spontaneous change to methæmoglobin. Hæmatoporphyrin also fails to permit growth, but hæmatin allows a copious growth.

(4) *B. influenzæ* on suitable media grows fairly well under anaërobic conditions, but rapidly dies out. The necessary oxygen is obtained from an oxygen store in the medium. This store is not dissolved active oxygen, but is inactive in a state of loose combination with a constituent of the medium. Under aërobic conditions the combined oxygen is used up by the bacillus and immediately re-formed from the atmosphere.

(5) The combined oxygen in the medium is activated for the bacillus by the catalytic action of iron in the pigment.

(6) The feebleness of the growth on unchanged blood compared with that upon changed blood is due to a deviation of the oxygen, activated by the iron of the pigment, from the bacillus through the oxygen affinity of the unchanged pigment.

REFERENCES.

1. FILDES, P.—"A New Medium for the Growth of *B. influenzæ*," *Brit. J. Exper. Pathol.*, 1920, **1**, 129.
2. PFEIFFER, R.—"Die Ætiologie der Influenza," *Zeitschr. f. Hyg. u. Infektionskrankh.*, 1893, **13**, 357.
3. CANTANI, A., JUN.—"Ueber das Wachsthum der Influenzabacillen auf hämoglobinfreien Nährböden," *ibid.*, 1901, **36**, 29.
4. GHON, A., AND V. PREYSS, W.—"Studien zur Biologie der Influenzabacillus," *Centralbl. f. Bakteriol.*, Orig., 1902, **32**, 90; 1904, **35**, 531.
5. LUERSSEN, A.—"Beiträge zur Biologie der Influenzabacillus," *ibid.*, Orig., 1904, **35**, 434.
6. DAVIS, D. J.—"Hemophilic Bacilli: Their Morphology and Relation to Respiratory Pigment," *J. Infect. Dis.*, 1907, **4**, 73.
7. GRASSBERGER, R.—"Zur Frage der Scheinfädenbildung in Influenzakulturen," *Centralbl. f. Bakteriol.*, 1898, **23**, 353.
8. FILDES, P.—"The Technique of the Preparation of Culture Media Containing Albuminous Fluids," *Lancet*, 1917, **1**, 492.
9. GOTSCHLICH, E.—"Allgemeine Morphologie und Biologie der pathogenen Mikroorganismen," in Kolle and Wassermann's *Handbuch*, Jena, 1912, **1**, 91.
10. MCINTOSH, J., AND FILDES, P.—"A New Apparatus for the Cultivation of Anaërobic Micro-organisms," *Lancet*, 1916, **1**, 768. "An Improved Form of McIntosh and Fildes' Anaërobic Apparatus," *Brit. J. Exper. Pathol.* (shortly).
11. BERTIN-SANS, H., AND MOITESSIER, J.—"Oxyhématine, hématine réduite et hémochromogène," *Compt. rend. Acad. d. Sci.*, 1893, **116**, 401.
12. OLSEN, O.—"Ueber die Bedeutung des Blutes für das Wachstum des Pfeifferschen Influenzabacillus," *Centralbl. f. Bakteriol.*, Orig., 1920, **85**, 12.

NOTE.

In a current number of the *Centralbl. f. Bakteriol.*, Olsen ([12]) also has arrived at the conclusion that the action of the blood-pigment is catalytic from the fact that iron-containing pigments grow the bacillus and give a positive guaiacum reaction with peroxide of hydrogen, while pigments which do not contain iron do neither. He does not account for, nor discuss, the difference in growth observed in the presence of hæmoglobin and hæmatin.

HÆMAGGLUTINATION AND ITS MEDICO-LEGAL BEARING, WITH OBSERVATIONS UPON THE THEORY OF ISOAGGLUTININS.

H. SCHÜTZE, M.D.

From the Bacteriological Department, Lister Institute, London.

Received for publication November 14th, 1920.

THE precipitin test has long been accepted as a means of differentiating one species of blood from another. Should the specimen be a human one, it might, however, be of the greatest medico-legal importance to carry the investigation one step further, and ascertain the iso-agglutination group* to which the specimen belongs. The necessity of investigating the possibility of grouping according to Moss (1) blood which is already dry arose for the author in connection with a case where material, alleged to be blood supernaturally pouring from a holy picture, was found to react positively with anti-human precipitin serum, and it was desirable to exclude or include certain persons as possible sources of supply.

No work appears to have been done to establish the possibility of grouping blood that has dried, or to find out for how long after desiccation such grouping might be practicable, but that a serum reconstructed from freshly dried blood still contains hæmagglutinins is seen from the following experiment:

Experiment I.—One c.c. of a Group II blood dried overnight in a Petri dish was reconstructed the following morning by rubbing up the material in 1 c.c. of distilled water with pestle and mortar and centrifuging to get rid of the undissolved residue. The hæmoglobin-stained supernatant was then used, alongside of serum obtained from the same individual, for the agglutination of red cells of known type.

Serum.	Red cell.	Serum dilutions. $\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$
Group II (Fresh) . .	Group I . .	↕	↕	↕	↕	↕	+	—
	Group III .	↕	↕	↕	↕	↕	+	—
Group II (Reconstructed after 1 day) . .	Group I . .	↕	↕	↕	↕	+	—	—
	Group III .	↕	↕	↕	↕	+	—	—

(↕ = macroscopic clumping; + = microscopic clumping.)

* The reactions of the Moss groups are as follows:

Serum.	I.	II.	III.	IV.
Cell I . .	—	+	+	+
Cell II . .	—	—	+	+
Cell III . .	—	+	—	+
Cell IV . .	—	—	—	—

(+ = agglutination of red cell by serum.)

In these tests the serum dilutions are carried out with physiological saline; the red cell suspension is a saline one containing 1·5 per cent. sodium citrate, and of such a density that a tiny platinum loopful added to a capillary drop (about 0·01 c.c.) of serum gives a concentration found convenient for the reading of results. This method was found to be sufficiently accurate to ensure quantitative reliability. The glass slide holding such drops is placed in a moist chamber at room temperature. Reading usually followed after one hour.

Confronted, as one would be in practice, with an unknown quantity of blood either in dry scale form or soaked into some material, one would have to arrive at quantitative measurements by working from a weighed amount of the dry blood, and reconstructing with sufficient distilled water to reproduce the volume of blood the dry sample represented.

Should such a reconstructed serum prove to contain no agglutinins, one would still have to decide whether the blood belonged to Group I, which possesses none, or whether its agglutinins had disappeared since the shedding of the blood. This decision might possibly be arrived at by establishing the character of the antigen presumably still existent in the undissolved residue spun down after reconstruction, by absorption of a Group IV serum with the residue and seeing what, if any, agglutinins were removed.

As a preliminary, absorption tests were carried out with fresh cells of the four groups (see Experiment II). Incidently, also, it was intended to see which of the theories, that of Moss [1], or that of Landsteiner [2] as amplified by Jansky [3] and later by v. Dungern and Hirschfeld [4], best fits the facts.

Moss, as is seen in the following table, originally put forward two explanations, the first of which he discarded on the strength of an absorption experiment, viz. the saturation of a Group IV serum with Group I cells, when he found that no agglutinins for either Group II or Group III cells remained, which result was incompatible with theory No. 1. Apparently he did not proceed to further cross-absorptions, so that Happ [5], in a recent paper also from the Johns Hopkins Hospital, still regards the second hypothesis as a possibility.

Moss's blood groups.	Moss's theory 1.		Moss's theory 2.		v. Dungern and Hirschfeld's theory.	
	Cell.	Serum.	Cell.	Serum.	Cell.	Serum.
I	A	—	A+B+C	—	A+B	—
II	B	a+c	B+C	a	A	b
III	C	a+b	A+C	b	B	a
IV	—	a+b+c	—	c	—	a+b

(Capital letters = antigens; small letters = agglutinins.)

If further absorptions had been performed, it would at once have been evident that Moss's second theory also was disproved.

Von Dungern and Hirschfeld's theory is simply an extension of that of Landsteiner to include Group I. This group, owing to its rarity, had been overlooked, and only the three other ones described. These workers assume the existence of but two antigens in the red cells of mankind—an antigen A, present in the cells of Group II individuals, and an antigen B in the cells of those of Group III. Both antigens are found together in Group I cells, while those of Group IV contain neither; these Group IV cells are devoid of agglutinable antigen. The

agglutinin content of the serum shows a curious reciprocity. Where there is a certain antigen in the cell, there the corresponding agglutinin is absent from the serum.

Group II has antigen A in the cell and agglutinin b in the serum, while Group III has B in the cell and a in the serum. As Group I cells contain both A and B, there is no agglutinin of either sort in the serum. Group IV, which has no antigen in its cells, has both agglutinins in its serum.

Von Dungern and Hirschfeld bring forward no absorptive evidence in proof of their theory, and are apparently convinced of its correctness by the simplicity with which it meets the facts of agglutination.

Experiment II.—Absorption in Experiment II was performed by mixing 0·4 c.c. serum with 0·4 c.c. of the centrifuged deposit of the washed red cells in question, the mixture standing at room temperature for 2 hours with intermittent shaking.

Serum IV.	Unabsorbed.								Absorbed with Cell I.							
	½	¼	⅛	1/16	1/32	1/64	1/128	1/256	½	¼	⅛	1/16	1/32	1/64	1/128	1/256
Cell I	‡	‡	‡	‡	‡	‡	‡	—	‡	+	—	—	—	—	—	—
Cell II	‡	‡	‡	‡	‡	‡	‡	—	‡	—	—	—	—	—	—	—
Cell III	‡	‡	‡	‡	‡	+	—	—	‡	+	—	—	—	—	—	—

Serum IV.	Absorbed with Cell II.								Absorbed with Cell III.							
	½	¼	⅛	1/16	1/32	1/64	1/128	1/256	½	¼	⅛	1/16	1/32	1/64	1/128	1/256
Cell I	‡	‡	‡	‡	+	—	—	—	‡	‡	‡	‡	‡	‡	+	—
Cell II	‡	—	—	—	—	—	—	—	‡	‡	‡	‡	‡	‡	+	—
Cell III	‡	‡	‡	‡	+	—	—	—	‡	—	—	—	—	—	—	—

Serum IV.	Absorbed with Cell IV.							
	½	¼	⅛	1/16	1/32	1/64	1/128	1/256
Cell I	‡	‡	‡	‡	‡	‡	+	—
Cell II	‡	‡	‡	‡	‡	‡	—	—
Cell III	‡	‡	‡	‡	+	—	—	—

From Experiment II it will be seen that while it is true that Moss' first theory is disproved by the fact that Group I cells remove from a Group IV serum the agglutinins for both Groups II and III as well as Group I cells, it is also true that his second theory cannot meet the facts of the experiment. Absorption of a Group IV serum with Group II or III cells does not remove the agglutinins for Group I cells as it would according to Moss, but, on the contrary, leaves them practically intact. On the other hand, it will be seen that the assumption that there are two substances, "A" and "B," in human red cells, with corresponding agglutinins, "a" and "b," in the serum, disposed in the manner indicated by v. Dungern and Hirschfeld, is sufficient to explain not only the agglutination phenomena but those of absorption as well.

Experiment III.—To test the possibility of grouping the residue from a

reconstructed blood, the centrifuged deposit left over from Experiment I was washed in saline, and re-suspended in 0·5 c.c. of the same fluid. Of this suspension 0·3 c.c. was added to 0·3 c.c. of Group IV (D)* serum, and the mixture allowed to stand for 2 hours at room temperature, being shaken from time to time. As the Group II cells (R) were known from previous experience to be less agglutinable than certain others also belonging to Group II, absorption of a second quantity of Group IV (D) serum was similarly carried out with the undissolved residue from the more agglutinable Group II cells (F), and agglutination demonstrated with both homologous and heterologous examples of well and less well agglutinating Group II cells. Corresponding absorptions, but with fresh washed cells (as in Experiment II) from the same persons, were undertaken at the same time.

Serum IV (D).	Unabsorbed.						Absorbed with Cell II (F).					
	½	¼	⅛	1/16	1/32	1/64	½	¼	⅛	1/16	1/32	1/64
Cell I (J)	‡	‡	‡	‡	‡	—	‡	‡	‡	+	—	—
Cell II (S)	‡	‡	‡	‡	‡	—	‡	—	—	—	—	—
Cell II (F)	‡	‡	‡	‡	‡	—	‡	—	—	—	—	—
Cell II (A)	‡	‡	‡	‡	+	—	—	—	—	—	—	—
Cell II (R)	‡	‡	‡	—	—	—	—	—	—	—	—	—
Cell III (Z)	‡	‡	‡	—	—	—	‡	‡	‡	—	—	—

Serum IV (D).	Absorbed with Residue II (F).						Absorbed with Cell II (R).					
	½	¼	⅛	1/16	1/32	1/64	½	¼	⅛	1/16	1/32	1/64
Cell I (J)	‡	‡	‡	+	—	—	‡	‡	‡	‡	+	—
Cell II (S)	‡	—	—	—	—	—	‡	‡	‡	‡	+	—
Cell II (F)	‡	—	—	—	—	—	‡	‡	‡	‡	+	—
Cell II (A)	—	—	—	—	—	—	‡	‡	—	—	—	—
Cell II (R)	—	—	—	—	—	—	‡	—	—	—	—	—
Cell III (Z)	‡	‡	‡	+	—	—	‡	‡	‡	—	—	—

Serum IV (D).	Absorbed with Residue II (R).					
	½	¼	⅛	1/16	1/32	1/64
Cell I (J)	‡	‡	‡	‡	‡	+
Cell II (S)	‡	‡	‡	‡	‡	—
Cell II (F)	‡	‡	‡	‡	‡	+
Cell II (A)	‡	‡	+	—	—	—
Cell II (R)	‡	—	—	—	—	—
Cell III (Z)	‡	‡	‡	—	—	—

It will be seen that absorption is as successful with blood residues as it is with fresh cells, and badly agglutinating cells are, as might be expected, also badly absorptive ones; but although saturation with such badly agglutinating Group II cells does not alter the titre of the serum for the better agglutin-

* Bracketed initials indicate the individual from whom the material was obtained.

ting Group II cells (S) and (F), the titre as registered by the more or less poorly agglutinating Group II cells (A) and (R) is definitely affected.

The recognition of the existence of poorly agglutinating Group II cells—(the cells of 5 individuals out of 13 of Group II have been found to possess this characteristic in more or less marked degree; on the other hand, those of 9 persons of Group III which have been tested were all equally agglutinable)—is of importance for two reasons. When a serum whose titre may from one cause or another have suffered is being grouped, it is obviously advisable to choose a cell with maximal agglutinating powers; while if cell-suspension or residue of unknown nature is being grouped by the absorption method, it is, on the contrary, better to employ a weak cell for agglutination, as being a better general indicator of an eventual decrease in the agglutinin content of the serum.

The possibility of grouping dried blood by agglutinating with its reconstructed serum and absorbing with the cell residue has been demonstrated, but it still remains to be seen how long the antigens and antibodies concerned remain diagnosable; this must no doubt depend on the original titre and the influences to which the dried blood has been subjected.

The present observations extend over a number of months, and grouping has been possible in every case.

In Experiment IV a low-titred blood is examined after 41 days' exposure to sunlight, the specimen being dried on cloth.

In Experiments V and VI high- and low-titred sera are examined 5 months after drying; in each case serum taken at the same bleeding and preserved bottled, in the cold room, is examined for an indication of the keeping qualities of hæmagglutinin sera.

Experiment IV.—Blood of Group III (St) was taken; 0·5 c.c. was dried on a piece of clean linen and exposed to the air on a shelf near a window, where much indirect and some direct sunlight fell upon it, and reconstructed as usual by rubbing and squeezing in a mortar, *but with* 1 *c.c.* of distilled water, after a period of 41 days.

Agglutination and absorption tests resulted as follows:

Serum III (St).	Fresh.				Reconstructed after 41 days on cloth exposed to sunlight.		
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$
Cell II (S) . .	‡	‡	‡	—	‡	‡	—

Serum IV (M).	Unabsorbed.					Absorbed with Residue III (St), reconstructed after 41 days.				
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$
Cell I (J) . .	‡	‡	‡	+	—	‡	‡	—	—	—
Cell II (S) . .	‡	‡	‡	+	—	‡	‡	+	—	—
Cell III (Sa) .	‡	‡	+	+	—	—	—	—	—	—

The titre has fallen slightly but the insoluble residue still shows marked avidity for its corresponding Group III cell agglutinins.

Experiment V.—In Experiment V blood of Group II (R) was taken; this individual constantly yields a high-titred serum with cells of poor agglutinability. One c.c. dried in a Petri dish was reconstructed and titred the following day; two similar specimens, one kept in a cupboard sheltered from light and the other exposed to it at a window, and both at room temperature, were reconstructed after 5 months, and examined together with a sample of the same bleeding kept, *as serum*, bottled and in the cold room.

The following titres were obtained:

Blood.	Particulars.	Titre against Cell I (J) and Cell III (Z).
II (R) undried . .	Serum at time of bleeding	$\frac{1}{64}$
,, dried . .	Reconstructed after 1 day	$\frac{1}{32}$
,, ,, . .	Reconstructed after 5 months, sheltered from light	$\frac{1}{8}$
,, ,, . .	Reconstructed after 5 months, exposed to light	$\frac{1}{4}$
,, undried . .	Serum after 5 months in the cold room	$\frac{1}{32}$

The five-months-old dried blood still retains sufficient agglutinin to make the grouping of it an easy matter; even the specimen freely exposed to sunlight, though of a lower titre than the one preserved in the dark, yields a serum capable of agglutinating Group I and III cells in a dilution of 1 in 4. The serum preserved for diagnosis purposes in the cold room has lost during the same period but little of its agglutinating power.

Experiment VI.—In Experiment VI, blood Group II (F) was used; the donor of this has, in contra-distinction to (R) of the previous experiment, always been found to have a low-titred serum with easily agglutinable red cells, but this inverse ratio of agglutinability of cell to titre of serum does not appear to hold good in all cases. One c.c. of Group II (F) was dried and reconstructed after one day; a further c.c. was dried, exposed to sunlight for 5 months and then examined; in both cases the absorption power of the insoluble residue was tested. The keeping qualities of a low-titred serum at 0° C. was also observed.

Blood.	Particulars.	Titre against Cell I (J) and Cell III (Z).
II (F) undried . .	Serum at time of bleeding	$\frac{1}{8}$
,, dried . .	Reconstructed after 1 day	$\frac{1}{4}$
,, ,, . .	Reconstructed after 5 months, exposed to light	$\frac{1}{1}$
,, undried . .	Serum after 5 months in the cold room	$\frac{1}{4}$

Serum IV (D).	Unabsorbed.						Absorbed with Residue II (F), reconstructed after 1 day.					
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$
Cell II (S) . .	‡	‡	‡	‡	‡	—	‡	—	—	—	—	—
Cell II (R) . .	‡	‡	‡	—	—	—	—	—	—	—	—	—
Cell III (Z) . .	‡	‡	‡	—	—	—	‡	‡	‡	+	—	—

Serum IV (D).	Unabsorbed.						Absorbed with Residue II (F), reconstructed after 5 months, exposed to light.					
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$
Cell II (S) . .	‡	‡	‡	‡	‡	‡	‡	‡	‡	‡	—	—
Cell II (R) . .	‡	‡	‡	‡	—	—	—	—	—	—	—	—
Cell III (Z) . .	‡	‡	‡	‡	—	—	‡	‡	‡	‡	—	—

The low-titred blood Group II (F) after 5 months' exposure to light in a desiccated condition has just sufficient agglutinating power left to indicate the group to which it belongs when tested in undiluted strength.

The complementary absorption test, however, establishes the group with more definiteness, although the specimen shows deterioration in this respect, too. Here again it is seen that less readily agglutinated cells, such as Group II (R), serve best as an indication of absorption. While the titre of the Group IV (D) serum is only somewhat lowered for the easily agglutinable cell Group II (S), that for cell Group II (R) is more markedly affected; the alien groups cells III (Z) agglutinate to the same extent in both absorbed and unabsorbed sera.

That portion of the Group II (F) bleeding kept, *as serum*, in the cold shows little depreciation in its agglutinating capacity.

It can be stated, then, that both antigen and agglutinin continue to be maintained for months in diagnosable form in dried blood specimens, and that probably in those blood-stains which have suffered most from adverse conditions, the absorption of a Group IV serum with the insoluble residue obtained when the dried blood is reconstructed will be the more successful diagnostic method.

With regard to the keeping properties of hæmagglutinating serum for diagnostic purposes, it may be mentioned here that the addition of 0·5 per cent. phenol, as is customary in the case of bacterial agglutinating sera, is not to be recommended. There is evidence that under the influence of the preservative the titre tends to sink.

Kolmer (6), working with serum, *not whole blood*, has found that samples dried on filter-paper deteriorate after 3 days in the cold room—how markedly one cannot tell, as the results given are qualitative only and not quantitative.

SUMMARY.

The possibility of grouping dried human blood specimens by reconstructing the serum for agglutination and using the undissolved residue for absorption has been demonstrated. Forensically the test would be of most importance when proving dissimilarity between two specimens alleged to be derived from the same source. To prove their similarity would probably only be to furnish circumstantial evidence of more or less value according to the group in question, and the frequency of the occurrence of that group in the population concerned.(7)

The Landsteiner theory that two substances, "A" and "B," with their corresponding agglutinins, "a" and "b," are concerned in the isoagglutination of human bloods has been confirmed by absorption tests.

REFERENCES.

1. Moss, W. L.—"Studies on Isoagglutinins and Isohemolysins," *Johns Hopkins Hosp. Bull.*, 1910, **21**, 63.
2. Landsteiner, K.—"Ueber Agglutinations-erscheinungen normalen menschlichen Blutes," *Wien. klin. Wochenschr.*, 1901, **14**, 1132.
3. Jansky, J.—"Hæmatologische Studien bei Psychotikern," reviewed in *Jahresb. f. Neurol. u. Psychiat.*, 1907, 1092.
4. Von Dungern, E., and Hirschfeld, L.—"Ueber gruppenspezifische Strukturen des Blutes," *Zeitschr. f. Immunitätsforsch.*, Orig., 1911, **8**, 526.
5. Happ, W. M.—"Appearance of Isoagglutinins in Infants and Children," *J. Exper. Med.*, 1920, **21**, 313.
6. Kolmer, J. A.—"The Influence of Desiccation upon Natural Hemolysins and Hemagglutinins in Human Sera," *J. Immunol.*, 1919, **4**, 393.
7. Hirschfeld, L. and H.—"Serological Differences between the Blood of Different Races," *Lancet*, 1919, **2**, 675.

THE PRESENCE OF HÆMOLYTIC SUBSTANCES IN HUMAN URINE.

ERIC PONDER, M.B.,

Assistant, Physiology Department, University of Edinburgh.

Received for publication November 6th, 1920.

In a paper published in 1915, C. S. McKee gave the results of his observations of the occurrence of hæmolytic substances in human urine. For the details of his technique, and for his results, readers are referred to his paper (1).

The writer was so placed as to have at his disposal a large number of cases of various forms of insanity, from which it was possible to select a considerable number who were in good health, and none of whom suffered from any of the diseases in which the presence of hæmolytic substances in the urine has been noted.

OBSERVATIONS ON THE FREQUENCY OF THE OCCURRENCE OF HÆMOLYTIC SUBSTANCES IN THE URINE.

(A) The hæmolytic titre of the urine was estimated in 30 cases; of these, 20 suffered from epilepsy and 10 from senile dementia. All were in good health. The technique of McKee was closely followed, except that, for his method of measuring quantities by drops, measurements in fractions of c.c. were substituted, on the basis that one drop from a capillary such as he described equals about ·03 c.c. The titre of a urine was taken as the minimum quantity which would completely hæmolyse 1 c.c. of the standard blood suspension, used by McKee, when the mixture was subjected to a temperature of 37° C. for 2½ hours. In each case the urine was examined in this way daily, the titrations being done at the same time each day (8 a.m).

The results may be expressed as follows:

In each of the 30 cases, at one time or another, hæmolytic activity was observed in the urine, no case giving persistently non-hæmolytic urine throughout the period during which it was under observation.

The daily variation in the hæmolytic titre was as a rule very marked; often for days the urine would fail to hæmolyse, and then, without an apparent cause, highly hæmolytic urine would be excreted.

The frequency with which the various titres of urine were met with is shown by the following table:

Cases.	Observations.	Percentage of total observations. Neg.	·45 c.c.	·36 c.c.	·30 c.c.	·21 c.c.	·12 c.c. or less.
8	336	54	15	13	10	2	6
12	336	75	13	10	1	·5	·5
10	210	71	11	4	4	8	2
Total 30	882	66·6	13	9	5	3·5	2·9

Each of the first group was examined on 42 consecutive days, each of the group of 12 cases on 28 consecutive days, and each of the group of 10 cases on 21 consecutive days.

Thus, out of 882 observations, hæmolytic substances were observed to be present on 33·3 per cent. of the occasions on which estimations were made, while no hæmolytic substance was detected on 66·6 per cent. of these occasions.

(B) Before making more extensive observations, it was considered advisable to investigate the technique used, to see if it gave the maximum number of positive results. Four points were specially investigated:

(1). *The time factor.*—The effect of the length of time in the incubator on the number of positive results obtained was investigated thus: 1000 urines, all known to be hæmolytic, were titrated by adding to various quantities of each urine 1 c.c. of the blood suspension, this being of a strength similar to that used above, but made with thrice washed cells. After one hour in the incubator at 37° C. the tubes were shaken; they were then returned to the incubator. After two hours the process was repeated, and the number of tubes showing hæmolysis noted. In a similar way the number of tubes showing hæmolysis at the end of 2½, 3, 4, 5 and 6 hours was ascertained, these numbers being expressed as a percentage of the total number of positives—1000.

Percentage positive after—					
2 hours.	2½ hours.	3 hours.	4 hours.	5 hours.	6 hours.
2·1	32·3	60·6	97	99·3	100

(No. of positives = 1000.)

It is, then, obvious that to state the number of urines which show hæmolytic activity at the end of 2½ hours is not to state the number which contains hæmolytic substances, but only about one-third of that number. This is one more instance of the neglect of the all-important time factor, and of the fallacy of assuming an end-point which does not exist experimentally.

(2) *Washing of cells.*—From the formulæ for the washing of precipitates (2) the quantity of serum left in McKee's suspension may be calculated; it will be found to be small. Since, as the result of research not included in this article, it has been found that this amount of serum may influence quantitative estimations considerably, the blood suspension used in the series of titrations below was made with thrice-washed cells, the resulting suspension being of greater sensitiveness than the suspension of once-washed cells.

(3) *Loss of hæmolytic power.*—The results of an investigation into the loss of hæmolytic power on standing may be summarised thus: There is a similarity in the way in which hæmolytic urines behave on standing, the difference being one more of degree than of kind. As a rule, a hæmolytic urine shows no loss of power in the first 12 hours after being passed. After this period loss of power is liable to occur; it commences in some urines sooner than in others, but proceeds with great rapidity once it has begun. It is occasionally, but not frequently, observed that a urine increases in hæmolytic power during the first few hours after being passed; this fact is recorded by McKee. It is therefore desirable to estimate the titre of a urine about 3 to 6 hours after it is passed.

(4) *Effect of temperature.*—There is no reason why this reaction should take place at body temperature only. Like most reactions, it is accelerated by high temperatures and retarded by low ones. The reaction occurs at 45° C., even more rapidly than at 37° C.; it also occurs at 30° C., the time taken being much longer. It is not proposed to fully discuss this question in this paper; it will be sufficient to note that subjection to incubator temperatures is not essential, but merely convenient.

As a result of these observations on technique, it was found necessary to devise a new method, of greater sensitiveness, for deciding the presence or absence of hæmolytic substances. The following was adopted:

Blood suspension.—To a tube containing 15 c.c. of citrated saline, add ·45 c.c. of blood drawn freely from the finger. Wash the cells thrice, using each time 15 c.c. of fluid, but using normal saline after the first washing. The washed cells are finally added to 50 c.c. of saline. The suspension is to be used soon after preparation.

Urines.—These are tested within a few hours of being passed. For each urine to be tested put up a series of 6 tubes containing 0·45 c.c., 0·36 c.c., 0·30 c.c., 0·21 c.c., 0·12 c.c., 0·06 c.c. of urine. To each tube add 1 c.c. of the blood suspension. Incubate for 4 hours at 37° C., shaking thrice in this time. Complete or marked hæmolysis to be considered positive. A few controls should always be put up.

(c) With this technique, a number of specimens of urine were examined, with a view to ascertaining the frequency of the appearance of the hæmolytic quality. These observations were made in three groups:

300 cases, examined on each of 14 consecutive days.—All were cases of various forms of insanity; all were in good health, cases with "organic" lesions being avoided. The urines were examined at the same time each day, some at 8 a.m. and some at 6 p.m. The results may be thus summarised:

No case gave negative results on more than 5 occasions out of the 14. Many cases gave no negative reading at all during the 14 days. There was a considerable diurnal variation in titre. Expressed in a tabular form, similar to that used in (A), we have the results:

		Percentage of total observations.					
Cases.	Observations.	Neg.	·45 c.c.	·36 c.c.	·30 c.c.	·21 c.c.	·12 c.c. or less.
200	2800	6·5	3·5	8	21	34	27

Thus out of 2800 titrations, hæmolytic activity was observed in 93·5 per cent., while only 6·5 per cent. were negative.

300 cases, observed on one occasion only.—These cases were all those of insane persons, all in good health. This series was examined from two points of view: (*a*) The numbers which showed various degrees of hæmolytic activity at the end of 4 hours; and (*b*) the numbers which showed hæmolytic activity at the end of 2½ hours.

(*a*) The results may be tabulated:

		Percentage of total observations.					
Cases.	Observations.	Neg.	·45 c.c.	·36 c.c.	·30 c.c.	·21 c.c.	·12 c.c. or less.
300	300	3·6	1·6	1·3	10·3	23	60

Or, out of 300 cases, 96·4 per cent. showed the presence of hæmolytic substances; in 3·6 per cent. these substances were absent. The 11 negatives were examined on subsequent days; none remained negative, but all showed hæmolytic activity.

(*b*) At the end of 2½ hours, of the 300 cases 47 per cent. showed hæmolytic activity, while 53 per cent. showed no activity. Combining the results of the 200 cases and of the 300 cases, we may express the result:

		Percentage of total observations.					
Cases.	Observations.	Neg.	·45 c.c.	·36 c.c.	·30 c.c.	·21 c.c.	·12 c.c. or less.
500	3100	5·1	2·6	4·7	15·5	28·5	43·5

Out of the 3100 titrations, 94·9 per cent. were positive, while 5·1 per cent. were negative.

The urines of 25 normal persons, titrated daily for 28 days.—The results may be summarised thus: No case showed a persistent negative reaction, each case showing, at one time or another, the presence of hæmolytic action. In tabular form:

		Percentage of total observations.					
Cases.	Observations.	Neg.	·45 c.c.	·36 c.c.	·30 c.c.	·21 c.c.	·12 c.c. or less.
25	700	5	3	13	9	25	45

Out of 700 titrations, 5 per cent. were negative and 95 per cent. positive.

From the observations on the urines the following conclusions may be drawn: Marked hæmolytic activity being observed at one time or another in 100 per cent. of the urines of insane persons examined and in 100 per cent. of the urines of normal persons examined, it is probable that the presence of hæmolytic action in the urine is a normal occurrence. In all probability it is due to a normal known urinary constituent.

THE PHYSICAL CONDITION OF HÆMOLYTIC URINE.

Two points suggest themselves for consideration:

(1) *The reaction of the urine.*—In all cases the reaction of the urine was tested qualitatively, while in a series of 50 cases the acidity was estimated by titration. The results may be summarised by saying that a hæmolytic action

may be shown by either an alkaline or an acid urine, although the majority of the hæmolytic urines examined were acid in reaction. The degree of acidity did not appear to exercise any effect. Certain of the persistently negative urines were alkaline; it was observed that an ammoniacal urine rarely produced hæmolysis. It has been pointed out (3) that solutions acid to P_H 5·1 produce hæmolysis; it is obvious that this has no bearing on the hæmolysis of urine (P_H 6 approximately) when such a urine is diluted 1 in 10.

(2) *The total solids.*—These are of importance, as they determine the tonicity of the urine. In a series of 50 specimens of urine, of various hæmolytic titre, the total solids were estimated; the osmotic pressure of the urine could approximately be calculated from this. It was found, in general, that urines with little hæmolytic power contained little solid, while highly hæmolytic urines tended to be more concentrated; the relation, however, did not seem to be a regular one. Many non-hæmolytic urines were found to be diluted specimens.

That the hæmolytic phenomenon observed in urine is not a result of a variation in tonicity is obvious from the following considerations: (1) That diluted specimens of urine which, when added to the saline suspension of blood, would tend to make it hypotonic, are, as a rule, non-hæmolytic, whereas urines which show hæmolytic power are, as a rule, those containing much solid, and which would tend to make the blood suspension to which they were added hypertonic; (2) that the addition of 0·1 c.c. of distilled water to 1 c.c. of the blood suspension will not cause hæmolysis, whereas the addition of 0·1 c.c. of a hæmolytic urine of a tonicity more equal to that of the blood will cause a rapid hæmolysis; (3) that the hæmolysis caused by urine is entirely inhibited by even a trace (0·005 c.c.) of blood-serum, or by the addition of lipoids, etc.

It is therefore necessary to consider that the hæmolysis is caused by some substance or substances, and not by a change in tonicity.

THE IDENTIFICATION OF THE HÆMOLYTIC SUBSTANCE.

Certain observations, which give an indication of the nature of the substance, may first be noted.

Raising a hæmolytic urine to a temperature of 100° seems to have no effect on its hæmolytic power; this temperature may be sustained for as long as an hour and a half without greatly reducing the activity of the urine. Further, the urine may be evaporated over a water-bath to dryness, and the residue treated with alcohol; the hæmolytic substance passes into the alcohol, which may be evaporated and the residue taken up with saline, giving a hæmolytic fluid.

In a similar way it may be shown that the substance is not soluble in ether, benzene or chloroform. It is obvious that it is fairly stable.

With a view to isolating the substance, two lines of research were followed: (*a*) a quantitative analysis of the constituents of hæmolytic and non-hæmolytic urines, and (*b*) an investigation into the hæmolytic activity of the various urinary constituents.

(*a*) Thirty urines were analysed, and at the same time their titres were

estimated. Chlorides, phosphates, oxalates, sulphur (organic, inorganic and neutral), urea, creatin, creatinin, uric acid, purin bases, hippuric acid, ammonia and total nitrogen were estimated quantitatively. Phenol, cresol, indol, indoxyl, skatol, aromatic oxyacids, leucin, cystin, tyrosin and nucleo-albumens were estimated by less exact methods.

This investigation threw no light on the question, the excretion of none of these substances being parallel to the hæmolytic titre.

Bile-acids and urobilin were also estimated; regarding these a detailed note is required.

Bile-acids.—The test used was that of Oliver. The urines were diluted 1 in 3 before testing. As soon as the peptone solution had been added the tubes were examined for a cloud; they were then set aside for 15 minutes, and again examined. The intensity of the cloud, and the time which it took to appear, were thus under observation. The tubes were examined both by transmitted light and against a dead black ground. In all there were tested 100 urines of known hæmolytic titre. It was found, without exception, that the urines which exhibited most hæmolytic activity gave the densest and most immediate clouds, while weakly hæmolytic urines, and urines which were non-hæmolytic, gave either scarcely a trace of cloud in the acid peptone or no cloud at all. So close was the parallelism between the hæmolytic activity and the cloud production in peptone, that it was found quite easy to foretell with accuracy the results which would be given by the titration of the hæmolytic power by examining the power of the urine to precipitate peptone.

Urobilin.—This was estimated by the spectroscope. The quantity of urobilin and the hæmolytic power ran roughly, but not absolutely, parallel.

(*b*) An investigation into the hæmolytic powers of the various urinary constituents did not reveal any substance of sufficient hæmolytic power to be considered as the cause of this occurrence, with the exception of the bile-acids and substances closely related to them. The bile-acids and their salts are very highly hæmolytic, a 1 in 10,000 solution of sodium taurocholate in saline, for instance, producing complete hæmolysis of a quantity of blood suspension considerably stronger than that used in the experiments above at 37° C., and in 4 hours. When this intensely hæmolytic power is considered, even a trace of such a substance in urine would be sufficient to confer on the urine a hæmolytic power.

That traces of bile-acids and their salts may occur in normal urine has been noticed by various observers (4). The results of the test applied for bile-acids, which are recorded above, point to this fact, no other substance which gives the peptone precipitation being known to occur in urine. It will also be observed that the results of attempts at extraction of the hæmolytic substance by various solvents show the substance to have a solubility similar to that of the bile-acids and their salts. It is further the case that these substances hydrolyse after a period of time, and on prolonged heating; thus, if the bile-acids were the hæmolytic agents, the loss of hæmolytic power on standing and the loss of power after prolonged heating would be explained.

It is therefore suggested that the hæmolytic activity of urine is due to

traces of bile-acids or of their salts; that this occurrence is physiological, and not necessarily connected with any abnormal process.

A note may be added on the inhibition of the hæmolytic action of urines which is caused by certain substances:

(*a*) *Sodium bicarbonate*, or a dilute alkali, will inhibit the hæmolytic action of urine; this fact was recorded by McKee.

(*b*) Minute quantities of serum have the same effect, but in a much greater degree, *e.g.* 0·005 c.c. of a certain normal serum inhibited the action of a urine whose titre was 0·27 c.c. Sera differ much in their inhibitory power; the question will not be dealt with fully in this paper.

(*c*) *Cerebro-spinal*, *ascitic* and other body-fluids have the same effect, but in a less degree.

(*d*) *Lipoids*, such as cholesterol and lecithin, and also certain fats, exert a powerfully inhibitory effect.

It is noteworthy that these same substances act as inhibitory agents to the hæmolytic action of the bile-acids and their salts, and that their inhibitory action on hæmolysis caused by urine is explicable on the ground that this hæmolysis is produced by the bile-acids and their salts.

This investigation is being continued in the Chemical Physiological Laboratory of Edinburgh University.

The writer desires to express his indebtedness to Prof. Sir Edward Sharpey Schafer for the interest which he has taken in this investigation, and for the encouragement and the opportunities which he has given. Dr. N. R. Phillips, Superintendent of the Monmouthshire County Asylum, Abergavenny, kindly gave every facility for the carrying out of the initial part of this investigation. To him especial thanks are due, and also to Dr. W. W. Taylor for the valuable advice and direction which he has given.

SUMMARY.

Hæmolytic activity was observed as a regular occurrence in the urines of insane persons, when tested for by a sensitive method. Such activity was also found to occur in the urines of healthy persons. It was found that in previous work on this subject certain important factors were overlooked. The hæmolytic substance appeared to be a stable chemical compound, capable of being extracted from the urine with various solvents. Its activity was inhibited by various substances, notably blood serum. There seems to be sufficient evidence to justify the suggestion that the hæmolytic power of urine depends on its containing minute traces of bile acids or their salts, and that the occurrence of hæmolytic activity in urine is physiological.

REFERENCES.

1. McKee, C. S.—"On the Hæmolytic Action of the Urine in Certain Conditions," *Brit. Med. J.*, 1915, **2**, 596.
2. Ostwald, W.—'Foundations of Analytical Chemistry,' 1908, p. 17.
3. Cole, S. W.—'Practical Physiological Chemistry,' 1915, p. 239.
4. Dragendorff.—*Zeitschr. f. anal. Chem.*, 1872, **11**, 467.

THE SACHS-GEORGI SYPHILIS REACTION AND ITS RELATION TO THE WASSERMANN REACTION.

TENJI TANIGUCHI.

From the Pathological Department of the University and Western Infirmary, Glasgow.

Received for publication January 6th, 1921.

Porges and Meier (1) and others have found that syphilitic sera caused precipitation of lecithin from a watery emulsion, as well as of sodium glycocholate. The phenomenon of precipitation has been held to be an essential antecedent to the absorption of complement in the Wassermann reaction: thus a number of precipitation reactions have been advanced as substitutes for the Wassermann reaction, the precipitable substances being lipoids or other hydrophilic colloids, or the attempt has been made to cause precipitation of the globulin of the serum, since it is agreed by numerous workers that the reacting power of syphilitic serum resides in the globulin fraction. But none of these reactions has come into practical use, as the general results did not run parallel either with the Wassermann reaction or with clinical diagnosis. Lately Sachs and Georgi (2, 3) have devised an improved method for detecting syphilitic infection by the formation of precipitates when the sera are mixed with cholesterolised alcoholic tissue extracts. The results show close parallelism to those of the Wassermann test and also correspond well with the clinical diagnosis. As this test is easier to carry out, it has been recommended by a number of workers as a substitute for the Wassermann reaction. It has been shown that physical conditions which are known to affect the Wassermann reaction exert a similar influence on Sachs and Georgi's reaction, *e.g.* the degree of dilution of the organ extract, the addition of cholesterol to the extract and the turbidity of the emulsion (Sachs and Georgi); also the reaction of the medium and the temperature, (Georgi) (4, 5). Accordingly, Sachs and his associates believe that the two reactions depend on the same principle. It has been found by a number of workers, however, that the two reactions are not strictly parallel either quantitatively or qualitatively; thus certain sera react positively to the Wassermann test but show a negative result in the Sachs-Georgi reaction, and *vice versâ*. As was pointed out by Neukirch (6) and Stilling (8), there often occurs non-specific precipitation when the mixtures of lipoid emulsion and human serum are allowed to stand at lower temperatures; accordingly Sachs and Georgi (1920) have modified their original procedure, and now keep the mixture for 18 to 20 hours at 37°C., making the reading immediately at the

end of this period (" incubator method "). In view of the importance of this reaction an investigation has been made as to the relationship between the Sachs-Georgi and the Wassermann reactions.

PARALLELISM BETWEEN THE WASSERMANN AND SACHS-GEORGI REACTIONS.

The Wassermann reactions have been carried out according to the following method employed in this laboratory [9]:

Antigen.—1 : 30 dilution of cholesterolised human heart alcoholic extract. *Hæmolytic system.*—3 per cent. suspension of ox corpuscles sensitised with excess of corresponding immune body (5 M. H. D.).

Procedure.—To 0·5 c.c. of antigen, 0·05 c.c. of heated serum (30 minutes at 56°C.) and then 2, 4, 6 M. H. D. of complement were added respectively for the first, second and third tubes. After the tubes had been incubated at 37°C. for 1·5 hours, 0·5 c.c. of sensitised ox corpuscles was added to each. Results were recorded after further incubation at 37°C. for one and a quarter hours and standing overnight at room temperature. *Controls.*—Serum alone, emulsion alone, also strong positive, weak positive and known negative sera. The results were recorded according to the method of Browning and Kennaway [10].

For carrying out the Sachs-Georgi reaction, the incubator method of Sachs and Georgi has been followed [3] except as regards the amount of serum used. According to Sachs and Georgi, one tube containing 0·1 c.c. of serum was used for the main test, but with this amount of serum a zone phenomenon occurs sometimes with extremely strong positive sera, as illustrated in Table I. It was also observed that a suspicious cloudiness may develop with certain negative sera in this amount; thus the one tube method with 0·1 c.c. of serum is not advisable in carrying out the reaction. Accordingly, for the main test at least two tubes containing 0·1 and 0·05 c.c. of serum were employed.

Antigen.—1·0 c.c. of 10 per cent. alcoholic human heart extract, as used in the Wassermann reaction, was diluted with 0·5 c.c. of absolute alcohol (practically equivalent to Sachs and Georgi's final dilution of extract); to one part of this diluted extract 0·05 c.c. of 1 per cent. alcoholic solution of cholesterol was added. Then 1 part of the cholesterolised extract was mixed with 5 parts of normal saline by the "double" method (viz. 1 c.c. each of extract and saline were mixed rapidly; after the mixture had stood for at least 15 minutes at room temperature, a further amount of 4 c.c. physiological saline was added rapidly). *Sera* (within 7 days after withdrawal of the blood) were tested after standing at least 3 hours at room temperature subsequent to heating 30 minutes at 56° C. (as recommended by Münster).

Procedure.—To each of two tubes containing 0·5 c.c. of emulsion, a volume of 1·0 c.c., consisting respectively of 0·1 and 0·05 c.c. serum diluted with saline, was added and mixed by slight shaking; the tubes were then incubated overnight (18 to 20 hours) at 37° C. *Controls* were similar to those used in the Wassermann reaction, for the serum control 0·1 c.c. of serum was used with 1·5 c.c. of saline containing the same amount of alcohol as in the test series (in the absence of alcohol a growth of bacteria often may be mistaken for

precipitate). Results were designated −, ±, +, ++, +++, ++++, respectively, indicating negative to strong positive precipitation.

In determining the result of the reaction, the presence or absence of precipitate in the second tube (containing 0·05 c.c. of serum) was principally taken into account (see above). But when this tube showed merely a suspicious reaction, the result in the tube containing 0·1 c.c. serum was compared with the controls; if this tube showed precipitation while the same tube in the negative control showed none, a positive result was recorded.

TABLE I.—*Illustrative Series showing Parallelism between Wassermann and Sachs-Georgi Reactions.* (The 7 sera detailed here were included in a series of 35, tested all on the same day.)

No. of serum.	Wassermann reaction.					Sachs-Georgi reaction.				
	Doses of complement.				Result.	Amounts of serum in c.c.				Result.
	2	4	6	serum control 2		0·1	0·05	0·03	serum control 0·1	
1	c	c	c	c	−	−	−	−	−	−
2	o	o	o	c	+	++++	+++	++	−	+
3	o	o	o	c	+	++	+	+	−	+
4	tr	m	jc	c	weak +	+	±	−	−	weak +
5	ac	c	c	c	−	±	−	−	−	−
6	ftr	tr	jc	c	weak +	*±	±	−	−	suspic.
7	o	o	o	c	+	†+	+++	++++	−	+
A (Controls)	d	vm	jc	c	weak +	+	±	−	−	weak +
B (Controls)	o	o	o	c	+	++++	++++	++	−	+
C (Controls)	c	c	c	c	−	−	−	−	−	−

Controls: For Wassermann reaction, M.H.D. of complement with 0·5 c.c. 3 per cent. ox corpuscles sensitised with 5 M.H.D. of immune body = 0·0075 c.c.; emulsion alone with two doses of complement = c; emulsion control for Sachs-Georgi reaction = no precipitation.

In the tables † indicates zone phenomenon; * indicates discrepancy. The degrees of hæmolysis in the Wassermann reaction are designated as follows: c = complete, jc = just complete, m = marked, d = distinct, tr = trace, ftr = faint trace, o = none. In the Sachs-Georgi reaction, the *plus* signs are in proportion to the degree of precipitation; − = no precipitation.

It is known that alcoholic extract of guinea-pig heart sometimes causes a false positive Wassermann reaction when used as antigen. The author [11] has demonstrated that this is due to the interaction between heterophile antibody contained in the particular normal human sera (normal sheep hæmolysin) and heterophile antigen contained in the guinea-pig tissue extract. An analogous fallacy in the Sachs-Georgi reaction has also been observed.

According to Sachs and Georgi, the precipitate obtained by the incubator method is less marked than that by the older method; but the author has observed that the results in the former case can be intensified by allowing the tubes to stand for 15 to 30 minutes at room temperature after removal

from the incubator; thus it is advisable in suspicious cases to repeat the reading after 30 minutes, in addition to the immediate reading after incubation; but, of course, the negative control must be observed likewise.

The results of the two reactions show very close parallelism; thus out of 296 cases examined on thirteen different days, 270 (91·2 per cent.) gave the same result, comprising 104 positives, 164 negatives and 2 suspicious; 26 cases (8·8 per cent.) showed discrepancies as detailed in Table II. None of the discrepancies can be regarded as serious, but it is to be noted that the largest proportion occurs in cases which are suspicious to the Wassermann test but negative to the Sachs-Georgi reaction.

TABLE II.—*Discrepanies between Sachs-Georgi and Wassermann Reactions in a total of* 296 *cases.*

	Results.					
Wassermann reaction .	Weak positive		Suspicious		Negative	
Sachs-Georgi reaction ..	—	?	—	weak +	weak +	?
Total 26	0	4	12	4	0	6

These results accord well with those of other investigators; thus the reactions were parallel in 94·94 per cent. out of 2770 cases (Sachs and Georgi); in 95·2 per cent. out of 165 (Plaut), in 85 per cent. out of 549 (Schröder). It seems unlikely that the discrepancies are due to any essential difference in the nature of the two reactions. In general, differences have been observed only in the case of weak or doubtful reactions. It is clear that the Sachs-Georgi reaction exhibits with certain sera a result intermediate between positive and negative, just as occurs with the Wassermann reaction (see Browning and Kennaway). Accordingly, here also the criterion of positive and negative stands on an empirical basis. As regards the relative advantages and disadvantages of the two tests from the practical standpoint, the Sachs-Georgi reaction suffers from the greater difficulty of distinguishing between negative and suspicious or weakly positive reactions, since slight differences in the state of aggregation of the suspension cannot be sharply distinguished; on the other hand, there is the advantage that the variable factor of the complement-containing serum is eliminated.

When the amount of complement fixed by the standard amount of serum in the Wassermann reaction is compared with the concentration of serum required to cause precipitation in the Sachs-Georgi test, it seems that a very close parallelism exists (Table III).

TABLE III.—*Parallelism between the Degree of Complement-fixation in the Wassermann Reaction and of Precipitation in the Sachs-Georgi Test.*

Series A.

For the Sachs-Georgi reaction in series A, the mixture of alcoholic lipoid and saline solutions was effected rapidly and all at one time in order to produce less turbid emulsions and so to facilitate accurate reading (the actual amount of precipitate being somewhat diminished thereby); in series B the antigen was diluted as described in the text. For the Wassermann reaction in both series cholesterolised extract was diluted in the proportion of 1 : 15 with saline. Excepting these modifications, the procedures for both reactions are the same as already described.

No. of serum.	Sachs-Georgi reaction: precipitate obtained with the following amounts of serum in c.c.— 0·1	0·05	0·03	0·01	Wassermann reaction: doses of complement fixed.
1	++++	++++	++++	+++	30+
2	++	+	±	—	20 to 25
3	+	+	±	—	15 „ 20
4	±	—	—	—	8 „ 10
5	±	—	—	—	6 „ 8
6	±	—	—	—	4 „ 6

In this series two other sera gave the same results as No. 3, and one other the same as No. 4.

Series B.

No. of serum.	Sachs-Georgi reaction: precipitate obtained with the following amounts of serum in c.c.— 0·1	0·05	0·03	0·01	Wassermann reaction: doses of complement fixed.
1	++	++++	++++	++++	40+
2	+++	++++	++++	++	35 to 40
3	++++	+++	+++	+	25 „ 30
4	+++	+++	+	±	15 „ 20
5	+++	++	+	—	12 „ 15
6	+	±	—	—	4 „ 6

In this series one other serum gave the same result as No. 4, and one the same as No. 6.

THE COMPARATIVE INTENSITY OF THE SACHS-GEORGI AND THE WASSERMANN REACTIONS WITH HEATED AND UNHEATED SERA; THE INFLUENCE OF COMPLEMENT ON THE PRECIPITATION REACTION.

It has been pointed out by Georgi [5] and by Münster [14] that, in contrast to the Wassermann reaction, unheated sera give rise to less intense

precipitation in the Sachs-Georgi reaction than occurs after inactivation at 56° C. This has been corroborated by Neukirch (7), who found also that fresh guinea-pig's serum possesses the property of diminishing precipitation when added to the mixture of lipoid emulsion and heated serum. Neukirch observed further that it causes solution of the precipitate, after it has been formed by the specific combination; the author has also found independently that fresh guinea-pig's serum lyses the precipitate obtained by adding the lipoids of heterophile antigen to the corresponding antibody. Again, these properties of guinea-pig's serum should be attributed to the action of complement; thus heated guinea-pig's serum is inactive in this respect, and lysis of the precipitate never takes place with fresh serum at 0° C. These differences in the behaviour of heated and unheated sera in the two reactions do not necessarily exclude the possibility of their being due to the same constituent. (See Table IV.)

TABLE IV.—*Lytic Effect of Fresh Guinea-pig's Serum on the Precipitate Formed in the Sachs-Georgi Reaction.*

A series of tubes containing lipoid emulsion and varying amounts of heated syphilitic sera were incubated at 37° C. overnight (Sachs-Georgi reaction): then 0·2 c.c. of guinea-pig's serum was added to each tube; in series (*a*) fresh (M.H.D. for 0·5 c.c. of 3 per cent. ox corpuscles sensitised with 5 M.H.D. of immune body =0·0075 c.c.), (*b*) heated for 30 minutes at 56° C. (0·2 c.c. caused no lysis of the test corpuscles), and the mixtures were kept at 37° C. for three hours. The results were as follows:

	Precipitate obtained with the following amounts of serum in c.c.		
	0·02	0·01	0·005
Before addition of guinea-pig serum . .	++++	+++	+
After addition of { fresh guinea-pig serum .	+++	+	−
After addition of { heated guinea-pig serum	++++	+++	+

THE NATURE OF THE PRECIPITABLE SUBSTANCE IN THE SACHS-GEORGI REACTION.

Highly divergent views are held regarding the part played by the lipoid emulsion in the formation of precipitates with syphilitic serum. Georgi (5) believes that the precipitate consists mainly of globulin derived from the syphilitic serum owing to the fact that it never redissolves completely in ether or alcohol. As is shown in the following example, however, the precipitate contains practically the total amount of lipoids of the emulsion. Thus when a lipoid emulsion is precipitated as completely as possible by adding a suitable amount of syphilitic serum until the supernatant fluid appears quite clear, it is found that an alcoholic extract obtained by treating the precipitate with an amount of alcohol equivalent to that originally present is practically as efficacious as the original extract for producing hæmolysis with cobra-venom; whereas the clear fluid, obtained after centrifuging off the precipitate, does not exhibit this property, except to a minimal degree.

Example.—To 3 c.c. of the same extract as was used in the Sachs-Georgi reaction, but without cholesterol, 15 c.c. of normal saline were added slowly to

produce an emulsion of maximum turbidity, and then 2 c.c. of syphilitic serum (amount of complement fixed by 0·05 c.c. of this serum with 0·5 c.c. of the lipoid emulsion diluted 1 : 15 = 40 + M. H. D.; maximum precipitating dose for 0·5 c.c. of test lipoid emulsion = 0·05 c.c.). The volume was then made up to 50 c.c., and 10 c.c. of the mixture were placed in a series of tubes, which were allowed to stand in the incubator at 37°C. for 24 hours, being shaken thoroughly at intervals. Thus a bulky precipitate formed and the supernatant fluid became clear. The precipitate was centrifuged at high speed and washed twice with salt solution (but a certain amount of the precipitate was lost owing to the difficulty in obtaining complete sedimentation). Finally the precipitate was extracted with 3 c.c. (original volume) of alcohol for 48 to 72 hours at room temperature, being shaken several times. The filtered alcoholic extract was tested for lytic power with cobra-venom with the following result:

Lytic dose for 1·0 c.c. of 5 per cent. ox corpuscles + cobra-venom 1 : 1000 in the different series:

(*a*) With emulsion of extract from precipitate (=1 : 8 dilution of original alcoholic solution, slowly mixed) = between 0·1 and 0·15 c.c.; [(*b*) with supernatant fluid (= 1 : 17 dilution of original alcoholic solution) 0·6 c.c. = trace of lysis]; (*c*) with emulsion of original extract (1 : 8 dilution, slowly mixed) = between 0·08 and 0·1 c.c.

CONCLUSIONS.

296 human sera were examined by the "incubator method" for Sachs and Georgi's reaction and simultaneously for the Wassermann reaction. 270 cases (91·2 per cent.) gave the same result with the two reactions, comprising 104 positives, 164 negatives and 2 suspicious; 26 cases (8·8 per cent. showed discrepancies. But none of the discrepancies can be regarded as serious, because the largest proportion (12 cases out of 26) occurs in the cases which are suspicious to the Wassermann test but negative to the Sachs-Georgi reaction, due apparently to the lesser delicacy of the latter reaction, owing to the difficulty of detecting slight precipitation. In general, differences have been observed only in the case of weak or doubtful reactions. Accordingly, it seems unlikely that the discrepancies are due to any essential difference in the nature of the two reactions.

When the amount of complement fixed by the standard amount of serum in the Wassermann reaction is compared with the concentration of serum required to cause precipitation there is also seen to be a close parallelism.

The difference in the behaviour of unheated sera in the two reactions does not necessarily exclude the possibility of their being due to the same constituent, as there is evidence that the thermolabile constituent of the serum, which is not a specific constituent in the syphilis reaction, affects differently the result in the two reactions.

The precipitable substance in the Sachs-Georgi reaction consists mainly of the lipoids contained in the emulsion.

REFERENCES.

1. Porges, O., and Meier, G.—"Ueber die Rolle der Lipoide bei der Wassermann'schen Syphilis-Reaktion," *Berlin. klin. Wochenschr.*, 1908, **45**, 731.

2. Sachs, H., and Georgi, W.—"Zur Serodiagnostik der Syphilis mittels Ausflockung durch cholesterinierte Extrakte," *Med. Klin.*, 1918, **14**, 805.
3. *Idem.*—"Beiträge zur Serodiagnostik der Syphilis mittels Ausflockung durch cholesterinierte Extrakte," *Arb. a. d. Inst. f. exper. Therap. . . . Frankfurt a/M.*, 1920, **10**, 5.
4. Georgi, W.—"Die Bedeutung der Extraktbeschaffenheit für die Ausflockung des syphilitischen Blutserums," *Zeitschr. f. Immunitätsforsch.*, Orig., 1918, **27**, 518.
5. *Idem.*—"Studien über Serum-Ausflockung bei Syphilis," *Biochem. Zeitschr.*, 1919, **93**, 16.
6. Neukirch, P.—"Ueber den Einfluss der Temperature und anderer Faktoren auf die Serumausflockung bei Syphilis," *Arb. a. d. Inst. f. exper. Therap. . . . Frankfurt a/M.*, 1920, **10**, 47.
7. *Idem.*—"Studien über die Sachs-Georgische Ausflockungsreaktion," *Zeitschr. f. Immunitätsforsch.*, Orig., 1920, **29**, 498.
8. Stilling, E.—"Zur Frage der Specificität beim serologischen Luesnachweis mittels Ausflockung nach Sachs und Georgi," *Med. Klin.*, 1920, **16**, 41.
9. Browning, C. H., and Watson, D.—'Venereal Diseases,' London, 1919, p. 302 (Oxf. Med. Public.).
10. Browning, C. H., and Kennaway, E. L.—"Suggestions for a New Criterion of a Positive Wassermann Reaction based on an Analysis of 2334 Quantitative Tests," *J. Hyg.*, 1920, **19**, 87.
11. Taniguchi, T.—"A Source of Fallacy in the Wassermann Reaction depending on Heterogenetic Antibody in Human Serum," *J. Pathol. and Bacteriol.*, 1920, **23**, 368.
12. Schröder, H.—"Ueber Ergebnisse der Ausflockungsreaktion nach Meinicke und Sachs-Georgi für die serologische Luesdiagnostik," *Med. Klin.*, 1919, **15**, 515.
13. Plaut, F.—"Die Sachs-Georgische Ausflockungsreaktion für Syphilis," *Zeitschr. f. d. ges. Neurol. u. Psychiat.*, Ref., 1919, **17**, 383.
14. Münster, M.—"Untersuchungen und Erfahrungen mit der Sachs-Georgischen Reaktion zur Serodiagnostik der Syphilis," *München. med. Wochenschr.*, 1919, **66**, 505.